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Dactylogyrus intermedius parasitism enhances *Flavobacterium columnare* invasion and alters immune-related gene expression in *Carassius auratus*

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ABSTRACT: The monogenean *Dactylogyrus intermedius* and the bacterium *Flavobacterium columnare* are 2 common pathogens in aquaculture. The objective of the present study was to examine the effect of prior parasitism by *D. intermedius* on the susceptibility of goldfish to *F. columnare* and to explore the potential immune mechanisms related to the parasite infection. A *F. columnare* challenge trial was conducted between *D. intermedius*-parasitized and non-parasitized goldfish. The *F. columnare* load in gill, kidney, spleen and liver were compared. The expression of immune-related genes (*IL*-1*β*2, *TNF*-*α*1, *TGF*-*β*, *iNOS*-*a*, *C3* and *Lyz*) in gill and kidney of *D. intermedius*-only infected and uninfected control fish were evaluated. *D. intermedius*-parasitized goldfish exhibited higher mortality and significantly higher loads (3051 to 537379 genome equivalents [GEs] mg^{-1}) of *F. columnare*, which were 1.13 to 50.82-fold higher than non-parasitized fish (389 to 17829 GEs mg^{-1}). Furthermore, the immune genes *IL*-1*β*2, *TNF*-*α*1, *iNOS*-*a* and *Lyz* were up-regulated while the *TGF*-*β* and *C3* were down-regulated in the gill and kidney of parasite-infected fish compared to the non-parasitized controls. The down-regulation *TGF*-*β* and *C3* was especially noteworthy, as this might indicate the suppression of the host immune functions due to the parasitism by *D. intermedius*. Taken together, these data demonstrate that parasite infection can enhance bacterial invasion and presents a hypothesis, based on gene expression data, that modulation of host immune response could play a role.

KEY WORDS: Bacterial load · RT-PCR · Monogenean · Columnaris · Tumor necrosis factor · Interleukin · Goldfish · Cyprinid

INTRODUCTION

In aquaculture systems, host–pathogen interactions are rarely one-on-one due to the incredible prevalence and diversity of pathogens (Shoemaker et al. 2008), and individual hosts are often co-infected with multiple disease agents (Xu et al. 2012b). However, most studies on host–pathogen interactions concentrate on a single infection. Studying multi-species infections is gaining more and more attention, as secondary or opportunistic infections may lead to serious disease with adverse consequences such as delay in diagnosis and treatment, which impedes host recovery (Griffiths et al. 2011, Eswarappa et al. 2012).

Over the past few years there has been increasing evidence that multi-species co-infections contribute to the severity of some infectious diseases, especially bacterial diseases (Busch et al. 2003). For example, Bandilla et al. (2006) demonstrated *Argulus coregoni*

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infection could increase the susceptibility of rainbow trout to *Flavobacterium columnare*. Recently, it was shown that parasitism by *Ichthyophthirius multifiliis* enhanced the host's susceptibility to the bacterium *Edwardsiella ictaluri* and induced more severe mortality of channel catfish (Xu et al. 2012c). The susceptibility of channel catfish to *Aeromonas hydrophila* infection and resulting mortality also increased if the hosts were previously infected with *I. multifiliis* (Xu et al. 2012a).

Although some studies have shown that parasitic infection can enhance bacterial invasion, the precise mechanisms of this phenomenon remain unknown. The possible pathways for such increased susceptibility might be direct. For example, the parasites might directly damage fish skin and/or gills (Buchmann & Bresciani 1997) or the parasite might act as a vector for a bacterial pathogen (Cusack & Cone 1986, Xu et al. 2012b). Additionally, Xu et al. (2012a) speculated that some parasites could enhance the bacterial infection indirectly via reducing the host's immune protection. However, only a few studies have investigated this aspect using immuno-physiological approaches, and up until now no studies on immune gene regulation have been done (Bowers et al. 2000, Jørgensen & Buchmann 2007).

The monogenean *Dactylogyrus intermedius* and the bacterium *F. columnare* are 2 common pathogens of goldfish *Carassius auratus*, and they can induce high mortality and heavy economic losses (Dove & Ernst 1998, O'Halloran 2000). *D. intermedius* is a common parasite infecting the gills of cyprinid fishes and causes a serious problem in fish culture (Buchmann et al. 1993, 1995). This parasite has 2 to 4 eyespots and 1 pair of large anchor hooks; it lives a direct lifecycle without an intermediate host (ehulková & Gelnar 2006). Heavily infected fish exhibit swollen and pale gills, excessive mucus production and accelerated respiration, which results in severe damage to the host (Wang et al. 2009). *F. columnare* belongs to the family *Flavobacteriaceae* and is the aetiological agent of columnaris disease. Columnaris disease shows erosion and necrosis of gills and skin of fish and, moreover, leads to osmotic and electrolyte imbalances and eventually to the death of the infected fish (Bader et al. 2003, Tripathi et al. 2005).

To our knowledge, there is currently no information available on the effect of prior *D. intermedius* parasitism on infection of the bacterium *F. columnare* in goldfish or whether the host immunocompetence is involved. Hence, the objectives of this study are to: (1) determine whether prior parasitism by *D. intermedius* would increase the infection load of *F. columnare* in different tissues (gill, kidney, spleen and liver) of goldfish; and (2) examine the expression of selected immune genes in gill and kidney of *D. intermedius*-infected fish to explore, at the gene level, whether the host's immune response to *D. intermedius* might contribute to *F. columnare* susceptibility.

MATERIALS AND METHODS

Fish and parasite samples

Four hundred healthy goldfish (mean weight 10.57 ± 1.83 g and mean length 7.85 ± 0.26 cm) were obtained from Shaanxi Fisheries Research Institute (Shaanxi, China) and maintained in a glass aquarium with 200 l aerated tap water (25.0 ± 0.2°C). Twenty goldfish were checked randomly to verify pathogen-free status of parasites (microscopy) and bacteria (plate methods and PCR). For this step we only focused on some frequently reported pathogenic bacteria: *Aeromonas hydrophila* (Wang et al. 2003), *Staphylococcus aureus* (Oliveira & de Lencastre 2002), *Vibrio harveyi* (Pang et al. 2006), *V. parahaemolyticus* (Yung et al. 1999) and the target *Flavobacterium columnare* (Panangala et al. 2007). Fish were acclimatized under laboratory conditions for 7 d.

The goldfish infected with *Dactylogyrus intermedius* were prepared following the methods described in our previous study (Wang et al. 2008). Briefly, healthy fish were cohabitated with those infected with *D. intermedius* for 3 wk, and thereafter, 10 fish were randomly killed and checked for the intensity of parasites under microscope.

Bacterial isolation and cultivation

An isolate of *F. columnare* (FC-G1) was obtained from the gills of diseased goldfish and cultured on Shieh agar plates (Shieh 1980, Decostere et al. 1997) at 27°C. The Shieh medium was composed as follows: peptone, 5 mg ml^{-1}; yeast extract, 0.5 mg ml^{-1}; sodium acetate, 10 µg ml^{-1}; $BaCl_2 \cdot (H_2O)_2$, 10 µg ml^{-1}; K_2HPO_4, 100 µg ml^{-1}; KH_2PO_4, 50 µg ml^{-1}; $MgSO_4 \cdot (H_2O)_7$, 300 µg ml^{-1}; $CaCl_2 \cdot (H_2O)_2$, 6.7 µg ml^{-1}; $FeSO_4 \cdot (H_2O)_7$, 1 µg ml^{-1}; $NaHCO_3$, 50 µg ml^{-1}; Noble agar, 10 mg ml^{-1}; distilled water, 1000 ml. This isolate of *F. columnare* was identified on the basis of colony morphology, Gram stain, standard biochemical tests (Shewan et al. 1960) and 16S rDNA sequence alignment (Weisburg et al. 1991). Single colonies were

picked from an agar plate and transferred to Shieh medium, cultured at 27°C under constant shaking for 96 h and used to challenge fish. The concentration (colony-forming units per milliliter, CFU ml^{-1}) of *F. columnare* was determined through serial 1:10 dilutions using standard plate counts.

Water quality

During trials, the water temperature, pH, salinity, and dissolved oxygen (DO) in tanks were detected with a thermometer, digital pH meter, refractometer, and portable oxygen meter, respectively. The actual concentrations of ammonia-N and nitrite-N in test solutions were determined using a portable ammonia analyzer (Sensidyne).

The mean ± SEM of temperature was 25.0 ± 0.2°C, pH was 6.9 ± 0.4, salinity was 20 ± 1 ppt, and OD was 6.9 ± 0.9 mg l^{-1}. Concentrations of ammonia-N and nitrite-N levels were 0.73 ± 0.21 mg l^{-1} and 0.11 ± 0.02 mg l^{-1}, respectively.

Experimental design and bacterial challenge

A total of 192 goldfish were chosen for the test, including 96 goldfish infected with parasites (Group A) and 96 non-infected goldfish (Group B). Both groups were divided into 8 tanks (45 × 30 × 25 cm, 15 cm water level) with 12 fish per tank. For the bacterial challenge, 4 tanks (48 fish) in each group were used. One hundred ml bacterial suspension (3×10^7 CFU ml^{-1}) was added to a 2 l glass beaker filled with 1 l water and then fish (groups with and without *D. intermedius*) were exposed in separate beakers to *F. columnare* for 1 h with aeration at room temperature (~25°C). The remaining 4 tanks of fish (48 fish) in each group were used to determine the immune genes expression in uninfected fish and in fish infested with *D. intermedius* only. These 96 fish were not exposed to *F. columnare* but kept in beakers with the same volume of water and with the same amount of Shieh medium for the same time. After challenge, all fish were returned to fresh tap water with aeration. Three tanks of fish in each treatment were monitored for mortality daily for 1 wk. At Hour 2, Day 1, Day 3 and Day 7 post *F. columnare* challenge, 3 fish from each group exposed to *F. columnare* were sampled to quantify *F. columnare* in different tissues (gill, kidney, spleen and liver). At the same sampling points, the gill and kidney from 3 fish in parasite infected group (not exposed to *F. columnare*) were collected to examine the immune genes expression. Six random uninfected fish (not exposed to *D. intermedius* or *F. columnare*) were used as a control group (Day 0). All tissues were sampled using aseptic technique after fish were anesthetized with an overdose of MS-222 (Geruien). Tissues for bacterial quantification were directly stored at −80°C while tissues for gene expressions were first snap frozen in liquid nitrogen and subsequently put in TRIzol reagent (Invitrogen) stored at −80°C.

Preparation of genomic DNA from bacteria and fish tissues

The genomic DNA (gDNA) of *F. columnare* (FC-G1) and other bacteria, used for generating a calibration curve to determine *F. columnare* levels in infected tissue, and/or to test specificity of *F. columnare* QPCR assay, were extracted and purified using the DNeasy Tissue kit (Qiagen) following the manufacturer's instructions. DNA yield and purity were determined spectrophotometrically using Nanodrop ND-1000. For standards, 10-fold serial dilutions from 10 ng μl^{-1} to 100 fg μl^{-1} of *F. columnare* gDNA was made with sterile water or with tissue extracts (gill, kidney, spleen, and liver) from uninfected fish. The same amount of tissue DNA was used for each dilution (1 µl per reaction). The gDNA from fish (infected and uninfected from experimental challenge) tissues (~20 mg) were also extracted by the DNeasy Tissue kit (Qiagen) and eluted in 200 µl sterile water. The purified and concentrated gDNA was stored at −80°C until further use.

Bacterial quantification in different tissues

One-step quantitative real-time PCR (qPCR) analysis was performed using GenAmp 7300 Real-Time PCR Detection System (Applied Biosystems) and SYBR Premix Ex Taq II kit (TaKaRa). A pair of *F. columnare*-specific primers (AC, listed in Table 1) was designed for targeting a 91 bp amplicon of the chondroitin AC lyase gene of *F. columnare*. The primers were designed using Primer Premier 5 software and synthesized by Shanghai Sangon Biotechnology (Shanghai). Primers were evaluated for species specificity by a BLAST (National Center for Biotechnology Information, NCBI) search to determine homology to other known sequences in GenBank.

The qPCR assay was carried out in a final volume of 12.5 µl modified reaction mixtures based on Prid-

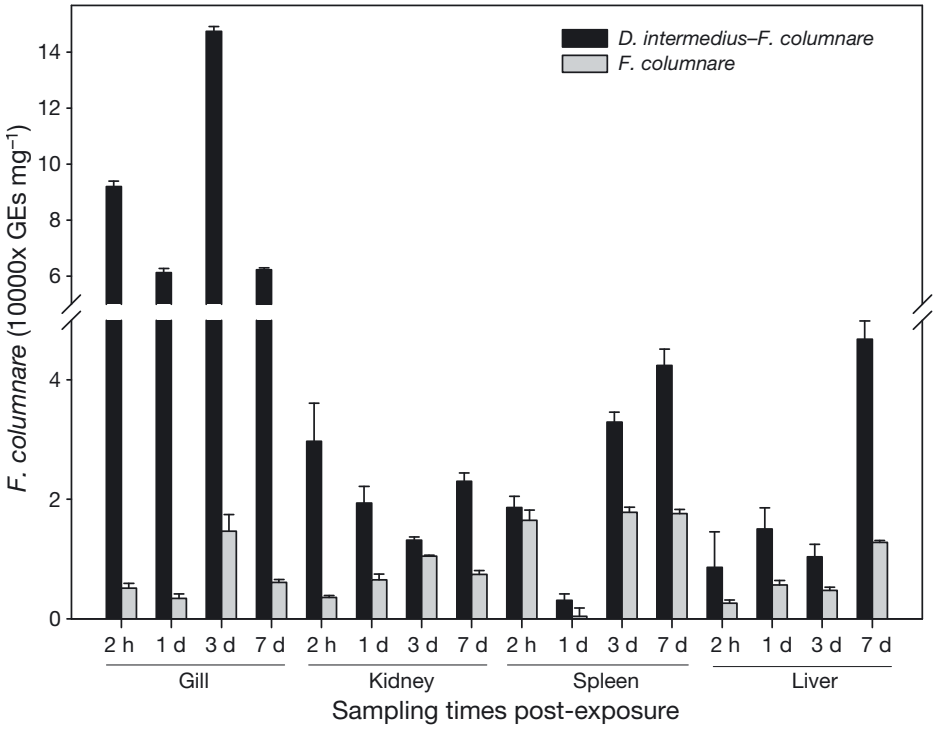

Fig. 1. Comparison of *Flavobacterium columnare* genome equivalents (GE) per mg of tissues between goldfish in Group A (coinfected with *Dactylogyrus intermedius* and *F. columnare*) and Group B (infected by *F. columnare* alone) at 2 h, 1 d, 3 d, and 7 d post bacterial exposure. *$p < 0.05$

geon et al. (2010) consisting of 0.5 µl of primer solution, 6.25 µl of Brilliant SYBR Green RT-PCR master mix, 1 µl of gDNA and 4.25 µl sterile MilliQ water. The parameters were as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 15 s, and 1 cycle of 72°C for 5 min. The quantification of *F. columnare* in fish tissues was measured via the standard curve (threshold cycle [C_T] values vs. DNA concentration of *F. columnare*). All qPCR reactions were run in triplicate for each sample. Since 1 µl of eluted sample was run in qPCR, the amount of bacterial DNA in each mg of tissue was equal to bacterial DNA concentration (pg µl^{-1}) × eluted volume/tissue weight in mg. Bacterial DNA in each mg of fish tissue was expressed as genome equivalents per mg of tissue (GEs mg^{-1}) based the 3.16 mbp genome size of *F. columnare* and a conversion factor of 1 pg = 978 mbp (Dolezel et al. 2003, Tekedar et al. 2012, Xu et al. 2012a).

Table 1. Sequences of primer pairs used in real-time PCR on goldfish *Carassius auratus*

Primer /target	Sequence	GenBank accession no.
AC	F: 5′-AAACACAATAACATTACCTGTACC-3′ R: 5′-TATCATAGATCATAGCTGATGCTC-3′	AY912281
β-actin	F: 5′-GATGATGAAATTGCCGCACTG-3′ R: 5′-ACCGACCATGACGCCCTGATGT-3′	AB039726.2
IL-1β2	F: 5′-GATGCGCTGCTCAGCTTCT-3′ R: 5′-AGTGGGTGCTACATTAACCATACG-3′	AJ249137.1
TNF-α1	F: 5′-CATTCCTACGGATGGCATTTACTT-3′ R: 5′-CCTCAGGAATGTCAGTCTTGCAT-3′	EU069817.1
TGF-β	F: 5′-GTACACTACGGCGGAGGATTG-3′ R: 5′-CGCTTCGATTCGCTTTCTCT-3′	EU086521.1
iNOS-a	F: 5′-TTGGTACATGGGCACTGAGATT-3′ R: 5′-CCAACCCGCTCAAGAACATT-3′	AY904362.1
C3	F: 5′-CTGTGCTGGCGGTTGT-3′ R: 5′-ATCCTCCATAATGAGACTGTTG-3′	AM773828.1
Lyz	F: 5′-GTTGTCCGTATCTTCAGGC-3′ R: 5′-CGCACTTTGTGGGTCTTA-3′	JN648715.1

Sensitivity and specificity of the qPCR assay

For sensitivity assays, the detection limit was evaluated from 100 ng µl^{-1} to 10 fg µl^{-1} of the gDNA of *F. columnare* in sterile water and in different tissue matrices (n = 4 reactions per dilution per matrix). The specificity of the PCR was determined by comparing PCR products derived from the following 11 cultures of bacteria: *F. columnare* (FC-G1) and other *F. columnare* isolates (G4; G18), *F. psychrophilum* (FP-1), *Aeromonas hydrophila* (2WCL-103), *A. caviae* (SX0841), *A. sobria* (HR07124), *Vibrio*

anguillarum (E-3-11), *V. fluvialis* (1.1609), *V. harveyi* (237), *Streptococcus* (PH-7), *Staphylococcus aureus* (C1231b). In addition, a melting curve analysis was performed to guarantee the specificity. Most of the bacteria used were isolated from diseased fish, identified to species using standard methods and stored in the Institute of Hydrobiology, Chinese Academy of Sciences (bacterial codes refer to isolates' reference numbers at the storage facility).

Expression of immune-related genes

Gill and kidney from goldfish not exposed to *F. columnare* and either uninfected or infected with *D. intermedius* were measured for immune-related genes expression. One piece of gill filament and a small piece of kidney were sampled and immersed in TRIzol reagent and stored at −80°C for RNA extraction. The rest of gill filaments were biopsied to determine the number of parasites. Total RNA of fish tissues was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA quality was verified by electrophoresis on ethidium bromide staining 1.0% agarose gels and by the absorbance ratio A_{260nm}/A_{280nm}. Afterwards, total RNA was reverse-transcribed into cDNA using the RevertAidTM First Strand cDNA Synthesis Kit (TaKaRa). The cycle consisted of 2 steps: 37°C for 15 min and 85°C for 5 s. Sequences of primers used for *β-actin*, *TNF-α*1, *IL-1β*2, *iNOS-a*, and *TGF-β* were obtained from previous studies (Oladiran et al. 2011, Lu et al. 2013), and primers for *C3* (complement C3) and *Lyz* (lysozyme) were designed using Primer Premier 5 software (Table 1). All the primers were designed across the splice cites to avoid genomic DNA contamination. *β-actin* was used as the housekeeping gene for normalization of expression levels from different samples. The cycle parameters for *TNF-α*1, *IL-1β*2, *iNOS-a*, and *TGF-β* were 95°C for 10 min followed by 40 cycles of 95°C for 5 s, 58°C for 20 s, and 72°C for 10 s; cycle parameters for *C3* and *Lyz* were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 54°C for 30 s. For each PCR run, a melting curve analysis was performed to guarantee the specificity in each reaction tube (absence of primer dimers and other nonspecific products). The reaction mixture included 6.25 µl of SYBR Premix Ex Taq^{TM}, 0.5 µl of forward and reverse primer (10 mM) and 1 µl of cDNA, and ultra pure water to a final total volume of 12.5 µl. Each individual sample was run in triplicate wells. RT-qPCR was performed using CFX96 Real-Time PCR Detection System (Bio-Rad).

Statistical analysis

The RT-PCR data were analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). Due to the relatively small number of fish sampled (n = 3) at each sampling time, non-parametric tests were used to analyzed the data. A Kruskal-Wallis test (KW) followed by a Dunn post-hoc test was performed to examine the differences in the expression of immune response genes between different times during the experiment. All statistical tests were performed using IBM SPSS ver. 21 software.

RESULTS

Parasite quantification, bacterial isolation and fish mortality

During the trial, the *Dactylogyrus intermedius* infestation intensity was moderate with a mean number of 46.7 parasites per fish (range: 35 to 59). No *D. intermedius* was observed in control fish. *D. intermedius*-parasitized goldfish exhibited much higher mortality (63.9%) when exposed to *Flavobacterium columnare* than non-parasitized fish (16.7%, Table 2). All sampled fish were positive for *F. columnare* in fish challenged with *F. columnare*, while no *F. columnare* was isolated from fish not exposed to *F. columnare*.

Sensitivity and specificity of primer and range of detection

Using the specific qPCR primers targeting the chondroitin *AC* lyase gene of *F. columnare*, amplified product was detected for the 3 *F. columnare* strains in qPCR reactions. The melting analysis showed there was only 1 melting peak, and only a single 91 bp amplified band was observed in all the 3 strains of *F.*

Table 2. Cumulative mortality and median days to death (MDD) of goldfish infected by *Flavobacterium columnare* and/or *Dactylogyrus intermedius* as well as control individuals. Shieh broth indicates 0 *F. columnare*. NA: not applicable

F. columnare (CFU ml^{-1})	*D. intermedius* mean no. per fish	No. of fish	No. dead	Mortality (%)	MDD
3×10^6	46.7	36	23	63.9	5.4
3×10^6	0	36	6	16.7	5.2
Shieh broth	46.7	36	0	0	NA
Shieh broth	0	36	0	0	NA

columnare by qPCR followed by gel electrophoresis. The 91 bp product was not observed in isolates of *F. psychrophilum*, *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, *Vibrio anguillarum*, *V. fluvialis, V. harveyi*, *Streptococcus* and *S. aureus*. No ambiguous bands were seen in these other species. No inhibition was observed in different tissue matrices.

A standard curve was performed with sterile water dilutions containing 50 ng to 50 fg of *F. columnare* gDNA. This allowed a linear correlation ($R^2 > 0.998$) between the chondroitin *AC* lyase gene amplicons from 1.55×10^7 to 15.5 copies and C_T values ranging from 18.38 up to 33.36, respectively. The regression equation between C_T values (y-axis) and log amount of nucleic acid (x-axis) was as follows: $y = -3.2331x + 41.301$, $R^2 = 0.9988$. The amplification efficiency in sterile water was 1.03. Also, the qPCR amplification efficiencies in different tissues indicated a high degree of efficiency in qPCR, with amplification efficiencies of 1.03 (gill), 0.98 (kidney), 0.99 (spleen) and 1.01 (liver).

Quantification of *F. columnare* in fish tissue samples

No *F. columnare* was detected in the screening of fish before exposure to *F. columnare*. The *D. intermedius*-parasitized fish showed 8581 to 260196 GEs mg^{-1} of *F. columnare* at Hour 2 in gill, kidney, spleen and liver, significantly higher ($p < 0.05$) than non-parasitized fish (2562 to 5120 GEs mg^{-1}) post *F. columnare* exposure (PFE). On Day 1, the bacterial loads in different tissues of parasitized fish ranged from 3051 to 106181 GEs mg^{-1}, significantly higher ($p < 0.05$) than that of non-parasitized fish PFE (389 to 6518 GEs mg^{-1}). The bacterial loads were significantly higher in gill, kidney, spleen and liver of parasitized fish (10366 to 537379 GEs mg^{-1}) than non-parasitized (4718 to 17829 GEs mg^{-1}) fish at 3 d PFE. The tissues of parasitized fish showed significantly ($p < 0.05$) higher numbers of bacterial (23034 to 110944 GEs mg^{-1}) than non-parasitized fish (6062 to 17601 GEs mg^{-1}) at 7 d PFE (Fig. 1).

Expression of immune-related genes in uninfected and *D. intermedius*-only infected fish

Gene expression in the gill

In the gill, the relative expression levels of all tested genes changed significantly over time in the parasite-infected group (KW test, *IL*-1β2 df = 4, p = 0.025; *TNF*-α1 df = 4, p = 0.012; *TGF*-β df = 4, p = 0.024; *C3* df = 4, p = 0.013; *Lyz* df = 4, p = 0.019), except for the *iNOS-a* (KW test, df = 4, $p > 0.05$). Specifically, a slight down-regulation of *IL*-1β2 was detected at Hour 2 and Day 1 followed by an obvious increment at Day 3 and Day 7 (Fig. 2A). No significant difference was found among different sampling times (Dunn test, $p > 0.05$ for all comparisons). In the case of *TNF*-α1, 3.36-fold and 2.73-fold increases were observed at Day 1 and Day 7, while it was only significant on Day 1 compared to the control group (Dunn test, $p < 0.05$, Fig. 2B). In contrast, *TGF*-β was down-regulated from Hour 2 to Day 7 in comparison to the control fish, and it showed statistical significance on Day 3 (Dunn test, $p < 0.05$, Fig. 2C). Expression of the *iNOS-a* was up-regulated in parasitized fish at all sample times, but not significantly (KW test, df = 4, p = 0.058, Fig. 2D). The expression levels of *C3* dropped dramatically at Hour 2 and remained low compared to the non-parasitized controls, exhibiting a significant difference on Day 7 (Dunn test, $p < 0.05$, Fig. 2E). Up-regulation of *Lyz* transcription was detected at Day 3 and Day 7 (2.33- and 3.19-fold increase, respectively, compared to control). The expression of *Lyz* was significantly greater only between Day 7 and Hour 2 (3.71 times higher on Day 7 PFE than at Hour 2 PFE; Dunn test, $p < 0.05$, Fig. 2F).

Gene expression in the kidney

In the kidney, the results showed significant differences in *IL*-1β2 expression over time during the experiment (KW test, df = 4, p = 0.012) with a significant increase between Hour 2 and Day 3 (1.67 times higher at Day 3 PFE than at Hour 2 PFE, Dunn test, $p < 0.05$, as illustrated in Fig. 3A). Similarly, the *TNF*-α1 increased significantly over the course of the experiment (KW test, df = 4, $p < 0.05$), although the increases were not significant among different sampling times (Dunn test, $p > 0.05$ for all comparisons, Fig. 3B). With respect to *TGF*-β, a successive down-regulation at Days 1, 3 and 7 was observed following an initial gentle increase at Hour 2, but the Dunn post-hoc test showed there was no significant difference between different groups (Fig. 3C). The expression of the cytokine *iNOS-a* was up-regulated at all sampling points, and on Day 7 it was significantly higher than that of the control group (Dunn test, $p < 0.05$, Fig. 3D). As in gill, a down-regulation of *C3* expression was observed at all sampling points, though no significant difference was found among time points (KW test, df = 4, p <

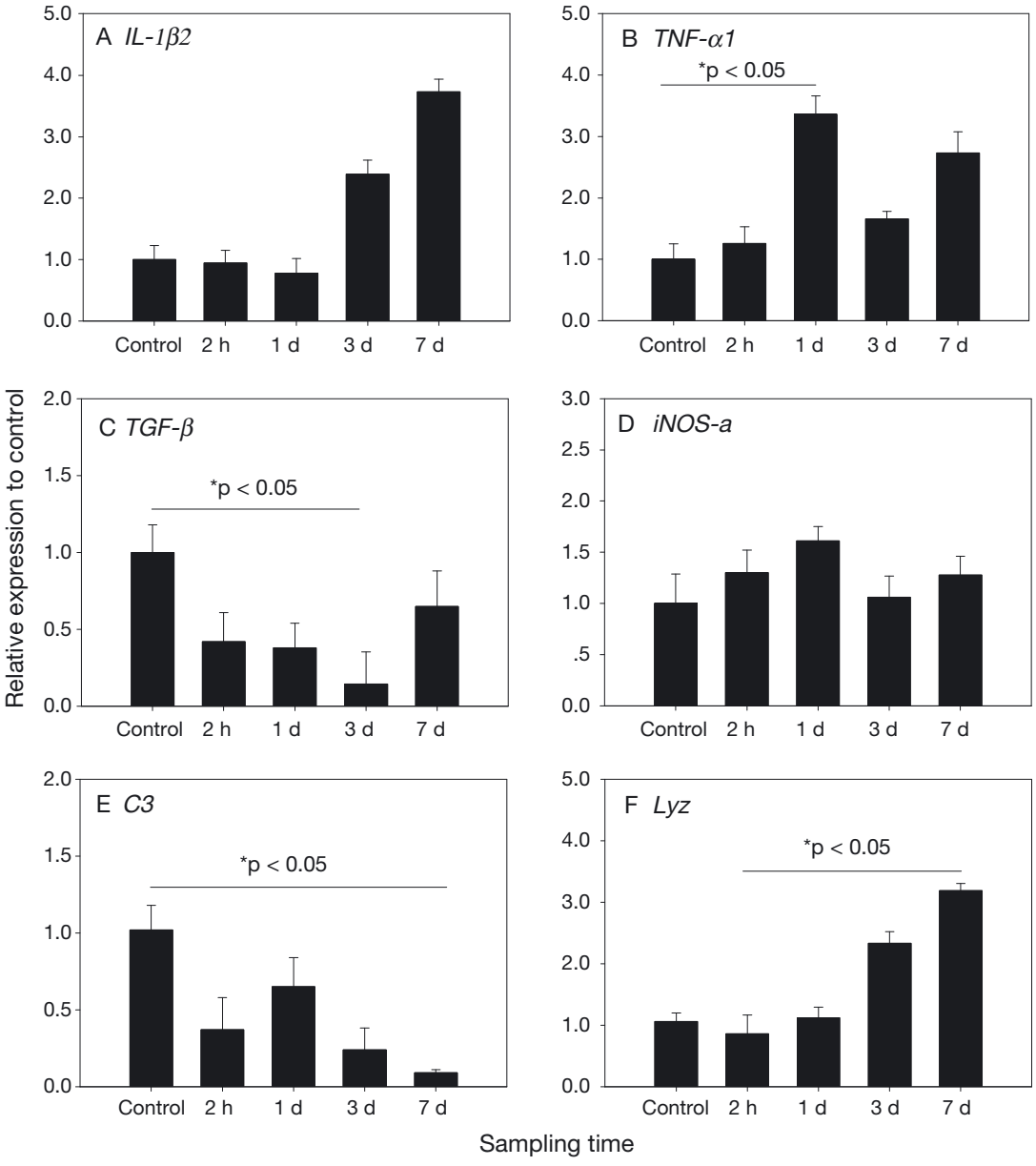

Fig. 2. RT-PCR amplification of immune-related gene expression in gill samples from *Dactylogyrus intermedius*-infected goldfish at 2 h, 1 d, 3 d and 7 d post bacterial infection. Bars represent mean ± SEM of 3 samples. *p < 0.05

0.05, Fig. 3E). The expression levels of *Lyz* was significantly greater in the parasitized infected group on Day 1 compared to the control group (3.58 times higher, Dunn test, $p < 0.05$, Fig. 3F).

DISCUSSION

Pathogen interactions could be performed as reciprocal facilitation, that is, Species A and Species B cross-feed on the byproducts of their partner; thus A enhances the growth of B and vice-versa (Pedersen & Fenton 2007, Eswarappa et al. 2012, Šimková et al. 2013). *Dactylogyrus intermedius* and *Flavobacterium columnare* are 2 common pathogens of goldfish, and until now there has been no study focused on whether prior infection by *D. intermedius* could promote *F. columnare* invasion and/or pathogenicity in goldfish. The current study demonstrated for the first time that infection by *D. intermedius* in goldfish significantly increased (1) the bacterial loads in different tissues following exposure to *F. columnare* and

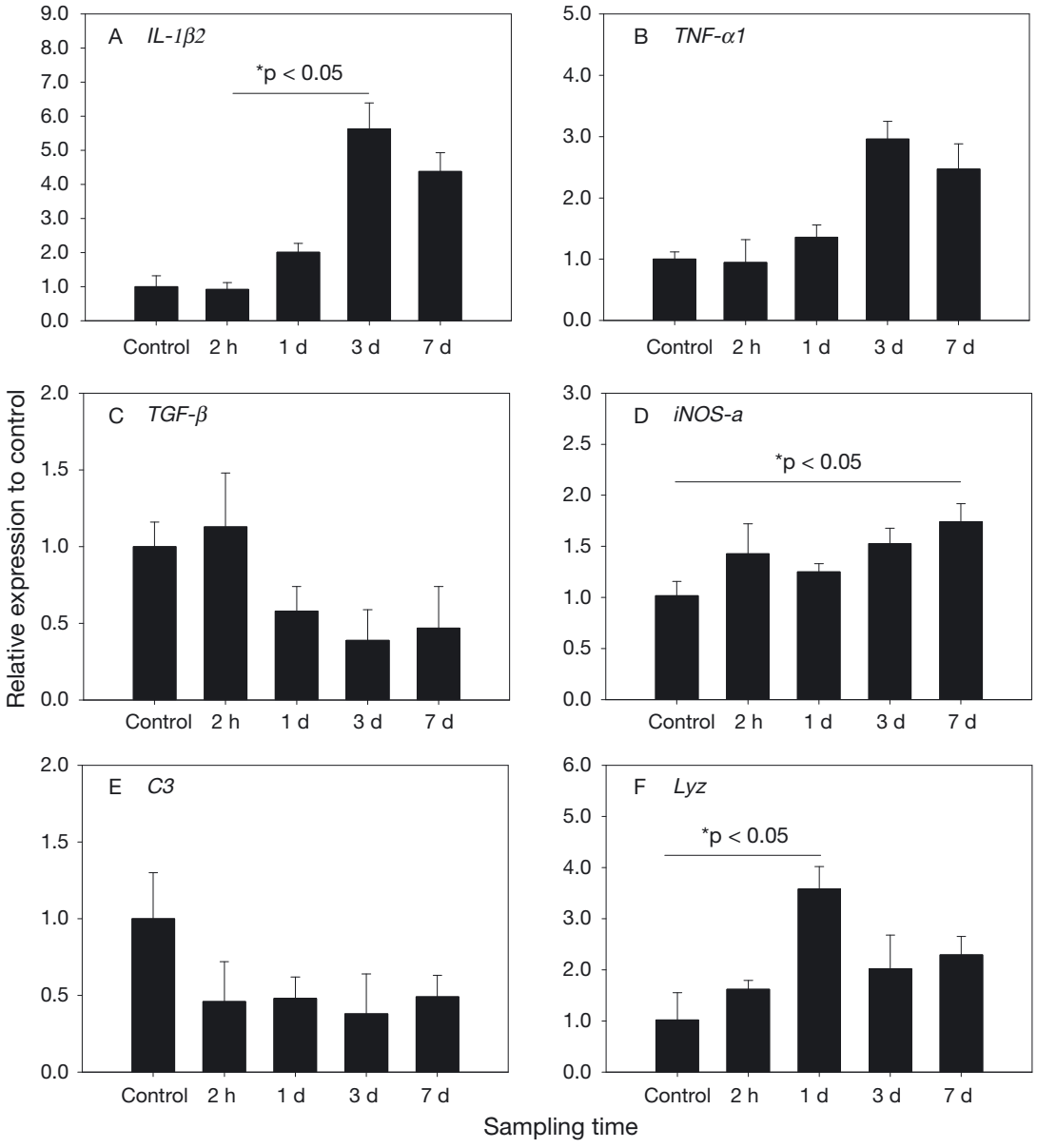

Fig. 3. RT-PCR amplification of immune-related gene expression in kidney samples from *Dactylogyrus intermedius*-infected goldfish at 2 h, 1 d, 3 d and 7 d post bacterial infection. Bars represent mean value (±SEM) of 3 samples. *p < 0.05

(2) mortality levels in the co-infected fish. The results of this study and previous studies (Evans et al. 2007, Martins et al. 2011, Xu et al. 2012a) have verified the hypothesis that prior parasite infection enhances the risk of bacterial infection.

This trial demonstrated that *D. intermedius*-parasitized fish exhibited a higher load of *F. columnare* in gill, kidney, spleen and liver than non-parasitized fish. The bacterial loads of parasitized fish ranged from 3051 to 537379 GEs mg^{-1}, which were roughly 1.13 to 50.82-fold higher than non-parasitized fish (with a bacterial load of 389 to 17829 GEs mg^{-1}). Furthermore, the gill tissue of parasitized fish exhibited the highest bacterial load among the 4 tested tissues at all observation points. This result was inconsistent with previous studies. Xu et al. (2012c) reported *Ichthyophthirius multifiliis* parasitism could increase the bacterial infection of *Edwardsiella ictaluri* and *A. hydrophila* in channel catfish, and the most bacteria were found in kidney from among many tissues sampled, including the gill. A suggested interpretation of the high amount of bacteria in the kidney might relate to the immune and blood filtering functions of the kidney (Russo et al. 2009). However, *F.*

columnare has been reported to mostly infect fish gills (Declercq et al. 2013), and therefore this might be the most probable reason for the highest load of *F. columnare* in the gills.

Nevertheless, our results may also reflect that infection dynamics might also be dependent on the types of parasites and bacteria. *D. intermedius* commonly parasitizes the gills of fish, causing gill damage, and *F. columnare* also occurs predominantly on the gills. In the present study, challenge with *D. intermedius* alone did not influence fish mortality. Some observational and experimental studies have described fish mortality due to different parasite species, infection doses and fish sizes (Menezes et al. 1990, Bandilla et al. 2006). The fish used in this trial carried a medium level of 40 parasites per fish, resembling the situation of some fish caught in nature, and this could explain why there was no mortality. However, it should be noted that although stress inflicted by low burdens of *D. intermedius* might not be manifested as mortality, other host life-history traits, e.g. growth rate or self-protection, may be negatively affected.

There are several possible pathways through which *D. intermedius* could increase the susceptibility of goldfish to *F. columnare*. Firstly, artificial abrasion could provide an efficient pathway for flavobacterial infections (Bader et al. 2003). The parasite *D. intermedius* might damage fish gills and therefore open a direct entrance for *F. columnare*. Secondly, *D. intermedius* might carry the bacterial pathogen *F. columnare* into the body of goldfish. A similar experiment showed the parasite *I. multifiliis* could transfer *E. ictaluri* to channel catfish (Xu et al. 2012b). However, more experiments to demonstrate the 2 assumptions are needed.

Apart from the 2 possible direct roles, another possible reason why *D. intermedius* increased the susceptibility of goldfish to *F. columnare* is that the parasite causes immune stress and reduces fish immune protective responses, thus increasing the ability of *F. columnare* to infect fish. Previous studies tried to verify this by measuring changes of the host's physiological or hematological indictors (Jørgensen & Buchmann 2007, Shoemaker et al. 2012). This present study attempted to confirm such a hypothesis at the gene expression level. Our results demonstrated that *TGF-β* and *C3* were both down-regulated in gills and kidney in *D. intermedius*-only infected goldfish. *TGF-β* is a well-known immunoregulatory cytokine and plays an important role in re-establishing immune homeostasis; it also maintains immunological balance, restricts the inflammatory reaction to the site of infection and prevents immune-mediated pathology (Faliex et al. 2008). *TGF-β* can also induce immune suppression in different types of cells or down-regulate some immune genes (Taylor et al. 2006). Complement 3 is the most abundant and important component in the complement system (Mosser & Edelson 1987, El-Lakkany et al. 2012) and is also regarded as possessing antibacterial activity (Saurabh & Sahoo 2008). The complement system plays an essential role in recognizing bacteria and can also lyse the bacterial cells, induce phagocyte migration and activate their phagocytic activity (Ellis 2001). Therefore, the decreased *TGF-β* and *C3* expression may have, to some extent, led to an immuno-suppressed state in fish. The down-regulation of these genes might play a pivotal role in the higher susceptibility to *F. columnare* in parasite-infected fish. We could assume that the down-regulation of *TGF-β* and *C3* was linked with *D. intermedius* infection. Our study and some former studies have shown that decreased immune related resistance in fish due to parasite stress could further increase fish susceptibility to bacterial infection (Pickering & Pottinger 1989).

In contrast to the down-regulation of *TGF-β* and *C3*, a slight up-regulation of *iNOS-a* was observed in the parasite-infected group, which is similar to results showing that *iNOS* isoforms increased in all organs in *Carassius auratus* infected with the parasite *Trypanosoma acarassii* (Oladiran et al. 2011). It is generally accepted that *iNOS* activates nitric oxide (NO) production during infections with bacterial and parasitic pathogens in fish, but the potential relationships of the elevated NO production and the higher *F. columnare* infection still need further study (Saeij et al. 2003). The precise reason for the up-regulation of *Lyz* is unknown, but it may be related to the mutual regulation of the immune genes. Previous studies have demonstrated that *IL-1β* possesses pleiotropic effects and its up-regulation may increase the lysozyme activity in the kidney of rainbow trout (Hong et al. 2003, Lindenstrøm et al. 2006). Our results for *IL-1β2* expression in both kidney and gills were consistent with the results of these previous studies, showing up-regulation over the experimental period with a similar upregulation of *Lyz* observed. Differences in immune gene expression levels in *D. intermedius*-only infected fish compared to controls, in particular at the early sampling points, may help explain increased susceptibility to *F. columnare* infection. However, it is unknown if expression at later sampling points is representative of how the response to *D. intermedius* evolves in the

combined infection. In addition, until *F. columnare* and *F. columnare + D. intermedius* groups are compared, other immune genes which may be suppressed by co-infection with *D. intermedius* may not be detected. It should also be noted that gene expression induced by parasites are species- and tissue-dependent; thus, further validation and extended studies of expression of additional genes in different organs among different hosts are still needed (Kania et al. 2007).

In summary, *D. intermedius*-parasitized goldfish showed significantly higher bacterial loads of *F. columnare* in gill, kidney, spleen and liver compared to non-parasitized fish after exposure to *F. columnare*. This study demonstrated that prior parasite infection could enhance bacterial invasion and result in higher fish mortality. Furthermore, the significant down-regulation of *TGF-β* and *C3* suggest that weakened immune functions of the host might be responsible (at least to some extent) for the increased bacterial susceptibility of the *D. intermedius*-parasitized goldfish.

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