
Investigations on susceptibility of marbled rabbitfish *Siganus rivulatus* to various infectious marine bacteria

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Abstract: Disease management in aquaculture is complicated by the fact that various fish species generally vary in their susceptibility to pathogenic microorganisms, thus requiring that every species be studied independently. The present work was performed to investigate the susceptibility of marbled rabbitfish *Siganus rivulatus* to the potential bacterial pathogens: *Aeromonas hydrophila*, *Mycobacterium marinum*, *Vibrio anguillarum*, *Streptococcus iniae*, and *Yersinia ruckeri*. Fish were challenged with the various bacteria and survival and hematological responses were evaluated. Mortalities in all treatments were not significantly greater than the control. However, analyses of hematological parameters suggest that *M. marinum* is potentially pathogenic to *S. rivulatus*. Additionally, we investigated whether lesions found on wild fish collected from Beirut beach contained any of the five bacterial species of interest. None of these bacteria were present in the lesions, thus suggesting that lesions were caused by other microbes.

Keywords: Rabbitfish, *Siganus Rivulatus*, Disease, Bacteria

1. Introduction

In 2010, global aquaculture production was approximately 60 million tons, accounting for nearly half of all fish consumed worldwide [1]. Further growth of the industry will require intensification of culture systems which would potentially increase infectious disease transmission. Such pathogenesis is an outcome of a complex interaction between the fish, the aquatic environment, and disease agents [2]. Accordingly, in order to develop adequate measures to control and prevent diseases, it is necessary to carry out multidisciplinary studies that would identify potential pathogens for each fish host and, subsequently, identify the role of environmental factors affecting disease in order to develop adequate measures to control fish health [3].

Siganids, also called rabbitfish or spinefoot are a family of Indo-Pacific herbivorous marine fishes with good aquaculture potential [4]. *Siganus rivulatus* are schooling fish that tolerate crowded conditions in captivity [5] and also tolerate broad ranges of temperature [6] and salinity [7]. Siganids, especially *Siganus rivulatus*, are in demand in the Middle East [8] which makes them economically valuable and interesting to

aquaculturists.

Infectious pathogens present in culture systems are usually also present in wild fish populations. However, diseases are more prevalent in culture systems because of stressful conditions such as high stocking density, poor water quality, inadequate nutrition, and injury in the mucosa, scales, or skin of the fish [3]. Moreover, some bacterial strains are primary pathogens that infect fish even with little stress while other opportunistic bacteria infect only heavily stressed fish [9]. Accordingly, understanding a microbe and its pathogenicity towards a fish species facilitates downstream steps that may eventually help in the development of methods to prevent, control, and treat disease.

Bacterial strains that are potentially pathogenic to rabbitfish are poorly studied but we know from anecdotal reports and personal observations that rabbitfish are affected by bacteria and can be treated with antibiotics. Accordingly, we chose to investigate the virulence of five bacterial species known to exist in the Mediterranean *Aeromonas hydrophila*, *Mycobacterium marinum*, *Vibrio anguillarum*, *Streptococcus*

iniae and *Yersinia ruckeri* [3], and to cause disease in marine fish [10].

Aeromonas hydrophila is a Gram-negative bacterium [11] that is an opportunistic pathogen [12]. When pathogenic, *A. hydrophila* causes a common bacterial disease called motile *Aeromonas* septicemia [13, 14]. Although *A. hydrophila* is generally a freshwater fish pathogen, it has been reported to infect marine fish species such as Atlantic salmon and Mediterranean Sea bass [15].

Mycobacterium marinum is a Gram-positive, straight or slightly curved bacillus [16]. *M. marinum* was found to be pathogenic to fish in an experiment performed on 50 poikilothermic species [17] and has been isolated in 20 marine and fresh water fish species so far [18]. In fish, the bacterium causes mycobacteriosis with symptoms such as emaciation and granuloma lesions and eventually death [19].

Vibrio anguillarum is a Gram-negative, rod-shaped bacterium [20]. Infection with *V. anguillarum* causes vibriosis, a severe condition characterized by deep focal necrotizing myositis, ulcerative necrosis of the body surface and sub-dermal hemorrhages at the base of the fins [21, 22]. The potential capability of *V. anguillarum* to cause huge fish losses in a variety of fish species [23] made it imperative for us to test whether this microorganism is capable of infecting rabbitfish.

Streptococcus iniae is a Gram-positive bacterium [24] that can cause skin lesions and necrotizing myositis upon infection [25]. *S. iniae* is considered a serious threat to aquaculture and a potential pathogen to rabbitfish.

Yersinia ruckeri is a Gram-negative, rod-shaped bacterium [26] responsible for enteric redmouth (ERM) disease and yersiniosis in salmonids [27]. Infection with *Y. ruckeri* is characterized by inflammation and redness in the mouth areas and throat [28]. The disease has also been reported in various marine fishes such as eels, sole, sturgeon and turbot [29]. Its pathogenicity in rabbitfish remains to be tested.

In the present work we assessed the virulence of these five piscine pathogenic bacteria to marbled spinefoot and studied the effect of bacterial challenge on hematological parameters. Additionally, we evaluated whether any of the five tested bacterial species existed in lesions in wild fish and hence might be implicated in rabbitfish disease.

2. Materials and Methods

2.1. Fish Acquisition and Holding

Juvenile marbled spinefoot (*S. rivulatus*) were caught in traps off the Beirut beach and transported live to the aquaculture research laboratory at the American University of Beirut (AUB). Fish were quarantined in two 1m³ tanks connected to a biological filter and a sump tank. They were offered a 35% protein commercial diet (Rangen EXTR 350, Rangen Inc., Buhl, Idaho) *ad libitum* twice daily. Fish were fasted for 24 hours prior to start of bacterial challenges.

2.2. Bacterial Cell Culture

Bacterial strains were obtained from the American Type

Culture Collection (ATCC, Manassas, Virginia, USA). Brain Heart Infusion (BHI) broth and agar were used to grow *Vibrio anguillarum* (ATCC19264), *Streptococcus iniae* (ATCC29178T), *Yersinia ruckeri* (ATCC29473), and *Aeromonas hydrophila* (ATCC43874). *Mycobacterium marinum* (ATCC92) was grown in Middlebrook 7H9 enrichment medium broth and on Middlebrook 7H10 enrichment medium agar. A standard optical density (OD) curve was developed for each bacterial species so that colony forming units (CFU) could be rapidly estimated when challenging the fish.

2.3. Experiment 1

Quarantined fish (8.64 ± 1.82 cm; 8.57 ± 3.53 g; mean \pm SD) with no symptoms of disease were randomly distributed into 18 plastic tanks (12 fish per tank) containing 5 L of filtered seawater and supplied with rigorous aeration. Bacteria were added to each container so that resulting numbers were approximately 10^7 CFU/ml and fish challenged for 60 min as described by [30] Crumlish *et al.* (2010). The virulence of each bacterial strain to the fish was tested in triplicate. Three tanks served as a control group where broth without bacteria was added. After exposure, fish were rinsed and placed in 52 L glass recovery tanks filled with filtered, chlorinated then de-chlorinated seawater for 10 days and counted twice daily. Twenty percent of the water in the tanks was changed daily and parameters were maintained at 37 ppt salinity, 25 °C and pH 8 and feed offered twice daily. Ten days after the challenge, blood was drawn out of three fish from each tank and hematological parameters assessed. Ammonia nitrogen and nitrite nitrogen were tested every other day and remained below 0.2 mg/L.

2.4. Experiment 2

Results of experiment 1 suggested that the bacteria tested were not pathogenic to marbled spinefoot at the concentrations tested. Consequently, an additional challenge was performed to confirm results. A strain of *V. anguillarum* known to be very virulent to trout and carp was obtained from the lab of Dr. Daniel Merrifield from the University of Plymouth. This strain in addition to the *V. anguillarum* used in experiment 1 and previously used strain of *M. marinum* were grown in broth. The challenge was performed in 500 ml glass beakers in order to challenge the fish with 10^8 CFU/ml of the bacteria. Fish were randomly distributed into 36 glass beakers (3 fish per beaker) each containing 250 mL of filtered seawater and supplied with aeration. Bacteria were added to each container so that resulting numbers were approximately 10^8 CFU/ml of each *V. anguillarum* (ATCC19264) and *M. marinum* (ATCC927) and the new strain of *V. anguillarum*. The virulence of each of the bacterial strains to the fish was tested in 9 beakers. After exposure, fish from three beakers were pooled, rinsed, and placed in 52 L glass recovery tanks so that 9 fish were present in each tank with three replicate tanks per treatment. Fish were maintained in the tanks for 10 days and counted twice daily. Twenty percent of the water in the tanks was changed daily and parameters were maintained as in

experiment 1.

2.5. Hematological Parameters

At the end of experiment 1, blood was taken from three fish per tank by cardiac puncture using heparinized syringes and was placed directly in heparinized tubes. Total red and white blood cells were stained with Natt-Herrick's solution [31] and counts were performed using a modified Neubauer hemocytometer. Slides for differential white blood cell counts were prepared by smearing a drop of fish blood on methanol-wiped microscope slides and stained with Wright-Giemsa stain. Eight hundred WBC were counted on each slide and cell counts were reported as percentage of total WBC count [32].

2.6. Identifying Bacteria in Fish Lesions

Sixty three wild adult marbled spinefoot were collected at the Ain El Mreisseh fishing harbor in Beirut. Four fish with lesions were found and their lesions swabbed: A lesion on tail with white spots, a lesion on upper lip, a caudal lesion with tail completely gone, and an abdominal lesion. Swabs were cultured in BHI broth and Middlebrook broth at three temperatures, 25 °C, 30 °C and 37 °C, and resulting isolates were stored in glycerol. BHI bacterial culture grown at 25 °C allowed for growth of *Y. ruckeri* and *V. anguillarum* present in the lesions. BHI bacterial culture grown at 37 °C allowed for growth of *A. hydrophila* and *S. iniae*. Middlebrook bacterial culture at 30 °C allowed for growth of *M. marinum* present in

the lesions. The various cultures were pelleted and DNA was extracted from the pellets followed by PCR amplification of species-specific genes such as N-acetylmuramoyl-L-alanine amidase [33], *lctO* [34], *hsp 65* [35] for *V. anguillarum*, *S. iniae*, and *M. marinum* respectively. The PCR cycling parameters were: denaturation step of 95°C for 3 min, followed by 33 cycles of 30sec at 95 °C, 1 min of appropriate annealing temperature for each primer pair and 1 min and 20 sec at 72 °C; then a final elongation step at 72 °C for 10 min. The band products of the lesions were compared to the band products of pure bacterial cultures of each of the five pathogenic bacteria being evaluated *A. hydrophila* (685bp), *Y. ruckeri* (589bp), *V. anguillarum* (429bp), *S. iniae* (870bp), and *M. marinum* (796bp) to identify whether the causative agent for disease was any of the five bacterial strains.

2.7. Statistical Analysis

Data was analyzed using SPSS statistical package (V.18 for Windows, SPSS Inc., Chicago, IL, USA) and reported as mean \pm standard deviation (SD) of the mean. Differences among survival means and hematological parameters were analyzed by one-way ANOVA, followed by Student Newman-Keuls means separation test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Fish Survival

Table 1. Fish survival 10 days after challenging *S. rivulatus* by adding the bacteria at a concentration of 107 CFU/ml to the culture water for one hour.

Bacterial Treatment	Number of dead fish/78	% Mortality	% Survival
Control	1	1.2	98.8
<i>A. hydrophila</i>	5	6.4	93.6
<i>V. anguillarum</i>	4	5.1	94.9
<i>Y. ruckeri</i>	2	2.5	97.5
<i>S. iniae</i>	3	3.8	96.2
<i>M. marinum</i>	0	0	100

Table 2. PCR primers used for each specific species, their sequences, target genes, annealing temperatures, and amplicon size.

Species	Primers	Primer sequence	Annealing temperature (°C)	Amplicon size
<i>A. hydrophila</i>	AH1	5'-GAAAGGTTGATGCCTAATACGTA-3'	62	685 bp
	AH2	5'-CGTGCTGGCAACAAAGGACAG-3'		
<i>Y. ruckeri</i>	YerF	5'-GCGAGGAGGAAGGGTTAAGTG-3'	62	589 bp
	YerR	5'-GAAGGCACCAAGGCATCTCTG-3'		
<i>V. anguillarum</i>	van-ami8	5'-ACATCATCCATTGTGTAC-3'	56	429 bp
	van-ami417	5'-CCTTATCACTATCCAAATTG-3'		
<i>S. iniae</i>	LOX-1	5'-AAGGGGAAATCGCAAGTGCC-3'	57	870 bp
	LOX-2	5'-ATATCTGATTGGCCGTCTAA-3'		
<i>M. marinum</i>	HS12F	5'-AGGGTATGCGGTTCGACAAG-3'	52	796 bp
	M2	5'-TTGAAGGCGATCTGCTT-3'		

In the first challenge experiment, there were no differences in survival among treatments (Table 1). Control tanks had 98.8% survival. Fish challenged with *A. hydrophila* had 93.6% survival and two fish exhibited some bloating and

lethargy. Fish challenged with *V. anguillarum* had 94.9% survival and two of the fish developed lesions suggestive of possible infection. Fish challenged with *S. iniae* had 96.2% survival and the only symptoms of disease observed were a

lesion in one fish and lethargy in a few fish during the first four days post challenge. Fish challenged with *Y. ruckeri* had 97.5% survival. Fish challenged with *M. marinum* showed no symptoms of disease and had 100% survival. It is noteworthy that most fish that suffered from lethargy did not die but rather regained vigor by the end of the first week. The little mortality observed happened on days 3, 4, and 5 post challenge. After day 5, no mortalities were observed in any of the treatments.

In the second experiment as well there were no significant differences in survival among treatments. Fish challenged with the same *V. anguillarum* strain as that used in the first experiment had 89% survival. Fish treated with the second *V. anguillarum* strain had 99.6% survival. None of the fish challenged with *M. marinum* died (Table 2).

3.2. Hematological Parameters

Table 3. Hematological parameters of rabbitfish *Siganus rivulatus* on day 10 post-challenge with five bacterial strains. Values with common superscript are not significantly different from each other. PSE = Pooled Standard Error.

	Parameter	Total RBC ($\times 10^6$ cells/ μ L)	WBC (cells/ mm^3)	Thrombocyte (%)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Eosinophil (%)
Control	Mean \pm SD	2.98 \pm 0.57 ^{ab}	10941.67 \pm 3235.87 ^a	86.4 \pm 1.47 ^a	10.6 \pm 3.02 ^a	0.69 \pm 0.61 ^{ab}	2.71 \pm 1.83 ^b	0.05 \pm 0.09 ^a
	Range	2.04-4.17	7375-17825	73-96	4-15	0-2	0-7	0
<i>A. hydrophila</i>	Mean \pm SD	2.87 \pm 0.61 ^{ab}	14645.83 \pm 4247.39 ^a	84.983 \pm 2.08 ^a	8.94 \pm 1.32 ^a	1.52 \pm 0.80 ^a	3.36 \pm 1.13 ^b	0.15 \pm 0.12 ^a
	Range	2.06-3.43	10950-22475	76-90	7-11	1-2	2-5	0
<i>V. anguillarum</i>	Mean \pm SD	3.04 \pm 0.39 ^{ab}	12779.17 \pm 3048.25 ^a	88.9 \pm 2.08 ^a	7.8 \pm 3.4 ^a	0.76 \pm 0.43 ^{ab}	3.27 \pm 1.47 ^b	0.07 \pm 0.10 ^a
	Range	2.38-3.46	7525-15900	84-94	4-13	0-1	2-5	0
<i>Y. ruckeri</i>	Mean \pm SD	2.78 \pm 0.51 ^{ab}	13212.50 \pm 5026.03 ^a	89.483 \pm 2.08 ^a	7.96 \pm 3.65 ^a	1.40 \pm 0.47 ^a	2.80 \pm 0.70 ^b	0.15 \pm 0.2 ^a
	Range	2.02-3.62	9450-22250	85-94	4-12	1-2	2-4	0
<i>S. iniae</i>	Mean \pm SD	2.56 \pm 0.40 ^b	11795.83 \pm 4050.17 ^a	87.467 \pm 2.08 ^a	8.75 \pm 3.21 ^a	1.4 \pm 0.28 ^a	2.92 \pm 1.07 ^b	0.12 \pm 0.11 ^a
	Range	2.03-3.07	7075-19275	83-92	5-14	1-2	1-4	0
<i>M. marinum</i>	Mean \pm SD	3.47 \pm 0.60 ^a	11004.17 \pm 1240.20 ^a	89.35 \pm 2.08 ^a	8.45 \pm 3.19 ^a	0.35 \pm 0.45 ^b	12.50 \pm 10.01 ^a	0 ^a
	Range	2.86-4.43	8975-12550	81-94	5-13	0-1	0-22	0
PSE		215926.8	1471.7	2.084	1.257	0.222	1.519	0.047

Table 4. Absolute values for the differential WBC and thrombocyte counts for 42 rabbitfish *S. rivulatus* (6 of each treatment and 12 from controls) on day 10 post-challenge with five bacterial strains.

	Parameter	Thrombocyte (cells/ mm^3)	Lymphocyte (cells/ mm^3)	Neutrophil (cells/ mm^3)	Monocyte (cells/ mm^3)	Eosinophil (cells/ mm^3)	N/L*
Control	Mean \pm SD	9388.1 \pm 2569.9	1168.3 \pm 382.27	249.84 \pm 135.33	50.27 \pm 50.37	4.79 \pm 8.4	0.34 \pm 0.49
	Range	6097-14678	383-1664	76-555	27-145	0-22	0.07-1.45
<i>A. Hydrophila</i>	Mean \pm SD	10075.13 \pm 3817.73	923.63 \pm 242.62	408.71 \pm 247.34	163.2 \pm 62.84	14.67 \pm 14.85	0.36 \pm 0.17
	Range	6085-17380	613-1290	212-803	96-256	0-40	0.18-0.58
<i>V. anguillarum</i>	Mean \pm SD	11780.97 \pm 4696.09	999.24 \pm 454.32	407.55 \pm 160.81	101.23 \pm 62	5.31 \pm 8.23	0.53 \pm 0.38
	Range	8537-20433	410-1622	242-611	18-185	0-16	0.18-1.15
<i>Y. ruckeri</i>	Mean \pm SD	11397.33 \pm 2589.65	1058.78 \pm 619.32	354.19 \pm 118.63	177.45 \pm 83.49	21.14 \pm 30.9	0.4 \pm 0.19
	Range	6860-14178	401-1890	247-551	125-345	0-80	0.17-0.66
<i>S. iniae</i>	Mean \pm SD	12876.98 \pm 4118.19	1237.38 \pm 433.94	387.73 \pm 193.97	208.24 \pm 91.59	13.48 \pm 12.6	0.38 \pm 0.26
	Range	9581-20602	858-1987	234-700	121-375	0-27	0.12-0.79
<i>M. marinum</i>	Mean \pm SD	9811.49 \pm 1001.02	947.63 \pm 430.73	1302.44 \pm 978.41	36.22 \pm 44.85	0 \pm 0	2.02 \pm 2
	Range	8167-11010	464-1715	313-2295	0-104	0-0	0.29-4.26

*N/L: Neutrophil to lymphocyte ratio

3.3. Testing Lesions for Presence of Bacteria

Species-specific amplicons were detected from DNA extracted from pure bacterial cultures. However, none of these amplicons were detected in cultures originating from the fish lesions, irrespective of the growth temperature (Figure 1).

The five bacteria tested were not associated with the lesions on the fish.

4. Discussion

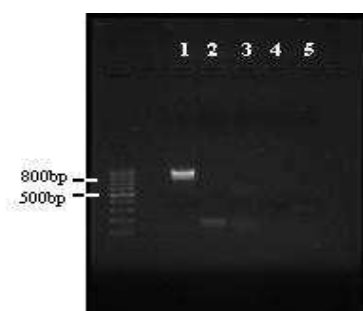


Figure 1. Agarose Gel Electrophoresis for PCR results for Middlebrook bacterial culture for all fish; Fish 1 (Column 2); fish 2 (Column 3); fish 3 (Column 4); fish 4 (Column 5) grown at 30°C show no presence of *M. marinum* DNA when compared to pure *M. marinum* DNA (Column 1).

The present work was performed to assess the susceptibility of the marbled spinefoot *Siganus rivulatus* to five known piscine pathogenic bacterial species. Results suggest that the marbled spinefoot is quite resistant to the bacterial strains used when challenged by adding the pathogens to the culture water. Fish in the present work were challenged with bacteria in the water because that is more relevant to aquaculturists. However, experiments have shown that siganids could be susceptible to bacterial infections when injected with the pathogen [36]. The reader should also keep in mind that information on bacterial diseases in rabbitfish is very limited and thus our discussion is limited to the present work with little to compare to.

Among fish exposed to *A. hydrophila*, one had a bloated stomach and died on day 3 post infection and another was lethargic until day five but recovered. The bloated stomach could be a sign of *A. hydrophila* infection but could also be the result of getting stung by another fish's spine during handling. Effects observed were not similar to those reported in the literature. The remaining fish in the *A. hydrophila* treatment appeared healthy and did not exhibit any signs of infection. When European eels *Anguilla anguilla* were challenged with *A. hydrophila*, a 17-34% mortality was observed along with symptoms of tail ulcerations and purulent liquid in the gills [37]. Whilst present results suggest that *Siganus rivulatus* are quite resistant to *A. hydrophila*, one should remember that *A. hydrophila* is an opportunistic pathogen and a lack of infection of fish could be because the fish used were healthy and in good physiological condition.

The *Vibrio anguillarum* challenge resulted in an average mortality of 5 %. Only one fish showed signs of disease and lethargy before dying; all other fish seemed healthy. In a

similar immersion challenge, visible external signs of vibriosis on coho salmon *Oncorhynchus kisutch* caused by *V. anguillarum* were reported [38]. A study found that rainbow trout *Salmo gairdneri* and saithe *Pollachium vireum* were susceptible to *V. anguillarum* isolated from conspecifics but that the bacteria were less pathogenic or non-pathogenic to fish species they were not isolated from [39]. Thus, the virulence of *V. anguillarum* is probably species-specific and a strain infectious to one species of fish might have no pathogenicity in another. In the present work, *S. rivulatus* appeared not to be affected by *V. anguillarum*.

In the *Yersinia ruckeri* challenge, only 2% of the fish died compared to 1% mortality in the control treatment; none showed any inflammation or redness in the mouth areas and throat, as expected from yersiniosis (red mouth disease). When challenged with *Y. ruckeri* under normal conditions, rainbow trout *Oncorhynchus mykiss* had 16.7% mortality [40] and Atlantic salmon showed 83% mortality and showed obvious yersiniosis [41]. Healthy marbled spinefoot appear to be resistant to *Y. ruckeri*.

Fish treated with *S. iniae* didn't develop any skin lesions which tend to be the typical sign of streptococcal infection. Although some fish were lethargic for a few days following the challenge, results are not significant because lethargy was observed in the other four treatments and the control. All fish regained energy and became active a couple of days following the challenge. In similar experiments using fish susceptible to *S. iniae*, such as sunshine bass, barramundi *Lates calcarifer*, and Japanese flounder *Paralichthys olivaceus*, excessive mortality was observed [42-44]. *Streptococcus iniae* was also found to be very pathogenic to the rabbitfish *Siganus canaliculatus* [45]. However, no mortality was seen when channel catfish *Ictalurus punctatus* were challenged with the bacterium [46]. Apparently, *S. iniae* does not affect all species similarly. Additionally, stress also appears to affect fish resistance to the bacterium. When non-stressed blue tilapia *Tilapia aurea* were challenged with *S. iniae*, no signs of disease were observed [47]. However, when the same fish were stressed by scraping off some scales then challenged with the bacteria, mortality increased to 90%. Accordingly, although fish in the present work were not affected by *S. iniae*, they may be susceptible if stressed.

Fish treated with *Mycobacterium marinum* had no mortality and didn't show any signs of Mycobacterium infection such as emaciation or lesions. When zebra fish, a model for *M. marinum* pathology was challenged with *M. marinum*, 10% of the fish died [48]. Survival results thus suggest that marbled rabbitfish are not susceptible to *M. marinum*. The second challenge with *M. marinum* and two *V. anguillarum* strains further confirmed this finding. It appears that even in stressful conditions and a greater concentration of bacteria, *S. rivulatus* is resistant to short term challenges with *M. marinum* and *V. anguillarum*.

RBC count was less in fish challenged with *S. iniae* than in control or any of the other treatments and greatest in fish challenged with *M. marinum*. Similar results were observed in

Coho salmon *Oncorhynchus kisutch* and in rainbow trout *Oncorhynchus mykiss* upon infection with *Vibrio anguillarum* [38, 49]. However, stressful conditions have also been shown to cause a significant increase in RBC counts [50]. Accordingly, we suspect that *M. marinum* challenge must have been stressful to *S. rivulatus* but the fish were able to fight off any infection in the ten days post challenge which are reflected in the increase rather than decrease in RBC counts. This analysis is supported by observations of neutrophil counts discussed below.

Upon infection, WBC counts of fish tend to increase [38, 51, 52]. Although WBC counts in the present work were not significantly different among treatments, the challenged fish had slightly greater counts than control fish. These results suggest that fish might have reacted to the challenges but because blood parameters were investigated ten days post challenge, any possible infection was already dealt with. Perhaps, had it been possible to measure hematological parameters only a couple of days post challenge, we would have observed an effect of bacterial challenge on WBC counts.

Differential thrombocyte and lymphocyte counts in our study did not reveal differences amongst treatments. In pacu *Piaractus mesopotamicus* challenged with *A. hydrophila* and rainbow trout infected with *V. anguillarum* the number of thrombocytes and lymphocytes in blood decreased 24 hours post-challenge [53, 54], suggesting migration of lymphocytes and thrombocytes to inflammation site and their role as defense cells at the onset of infection. Our experimental protocol did not allow us to test whether lymphocytes and thrombocytes were involved in helping *S. rivulatus* stave off infection. Because we measured these parameters on day 10 post-infection, we may have allowed thrombocytes and lymphocytes to return to their original quantities in the bloodstream even if they had decreased post challenge.

Blood of fish challenged with *Mycobacterium marinum* had significantly more neutrophils and significantly less monocytes compared to the control and other treatments. Previous work had reported increases in neutrophils and monocytes following bacterial infection [53-55] but we found no reports of increase in neutrophils and decrease in monocytes. Neutrophils and macrophages, which are the first responders to infection and develop from the same myeloid precursor stem cells as monocytes, kill microbes and bacteria through phagocytosis and are important components of the innate immune system [56]. An acute inflammatory response would be characterized by high numbers of neutrophils in blood and accumulation of neutrophils and macrophages at the site of injury or infection [57, 58]. Accordingly, it is possible that in the present experiment *M. marinum* affected the fish causing an increase in macrophages at the expense of monocytes that would originate from the same stem cells.

Bacterial DNA obtained from lesions on wild fish did not match any of the five known pathogens tested in the present work. No extra work was done on the samples because it is difficult to come to any conclusions on fish disease from these lesions. The history of the fishes was not known and the

lesions were found on only a few individuals in the wild population and on differing body parts. We know from experience that marbled spinefoot are susceptible to bacterial problems that can be treated with Florfenicol. We also know that stressed fish are very susceptible to disease. However, *S. rivulatus* is very tolerant of crowded conditions [5] and intensive farming does not cause stress. Anecdotal observations in our laboratory suggest that when exposed to low oxygen or high temperatures, the fish get diseased and die within a few days. Future work will investigate whether bacteria used in the present work become pathogenic when fish are stressed before or during challenge. The fact remains that non-stressed rabbitfish are quite resistant to common pathogenic bacteria, and are thus a good candidate for commercial aquaculture.

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