

A SURVEY OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS IN CULTURED SEA BASS AND ITS VIRULENCE ON JUVENILES OF SEA BASS, *DICENTRARCHUS LABRAX* (ACTINOPTERYGII: PERCIFORMES: MORONIDAE) AND GILTHEAD SEA BREAM, *SPARUS AURATA* (SPARIDAE)

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Background. Turkey is one of the major European seed producers of European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), and gilthead sea bream *Sparus aurata* Linnaeus, 1758. Determination of susceptibility to viral haemorrhagic septicaemia (VHS), a notifiable disease in Europe, is crucial particularly for sea bass and sea bream seed production to develop control measures and to take necessary actions in case of a VHS outbreak. We hypothesized that VHS virus does not replicate at 16°C and above—the temperature range typical for hatcheries in the Aegean region.

Materials and methods. To assess the seasonal occurrence and virulence of viral haemorrhagic septicaemia virus genogroup Ie (VHSV-Ie), a virological survey was conducted in cultured sea bass in the Black Sea. Thirty-five sea bass were sampled monthly from a local marine farm, and examined virologically. Triplicate groups of juvenile of sea bass ($n = 30$ per replicate or $n = 40$ per replicate) and gilthead sea bream ($n = 20$ per replicate) were challenged by immersion with VHSV-Ie at 12°C and 16°C to determine the occurrence of pathogen transfer and viral replication.

Results. VHSV-Ie, or any other viral pathogen able to infect BF-2 and CHSE-214 cells, did not occur in cultured sea bass of the Turkish Black Sea region. It was impossible to infect sea bass at 16°C but moderate levels of mortality occurred at 12°C. Sea bream, however, were susceptible to VHSV-Ie, presenting low levels of mortality (15%).

Conclusion. VHSV-Ie poses no risk to the production of sea bass seed carried out at 16°C and above, but sea bass and sea bream should be monitored for VHSV-Ie where ambient water temperature is below 16°C since the risk of introduction is present.

Keywords: seabass, sea bream, VHS, seed production, challenge, temperature, VHSE-Ie

INTRODUCTION

Viral haemorrhagic septicaemia (VHS), one of the principal viral diseases of cultured and wild fish, caused by viral haemorrhagic septicaemia virus (VHSV), which is a rhabdovirus belonging to the genus *Novirhabdovirus* (see Jensen 1965, Walker et al. 2000). More than 80 species of fish living in freshwater, seawater, and brackish water around the world were found to carry VHSV (Anonymous 2012). Many cases of severe VHS in wild and cultured fish were reported in North America and Europe (Wolf 1988, Meyers et al. 1999, Nishizawa et al. 2002), while most isolates were obtained from asymptomatic carrier fish (Skall et al. 2005).

Reported variations in the virulence of VHSV, isolated from various regions of the world, might be due to

genetic differences of the isolates. Nucleotide sequences and glycoproteins indicate four main, geographically distinct, genotypes (I to IV) of VHSV (Snow et al. 1999, 2004, Einer-Jensen et al. 2004, 2005). Freshwater isolates belong to either genotype I or IV, whereas marine isolates can be any of the four genotypes. Subgroup Ia represents freshwater VHSV isolates from European farms of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), while subgroup Ib represents marine isolates from the Baltic Sea, Kattegat, and Skagerrak. The isolates of subgroups Ib and Id were obtained from rainbow trout cultured in sea cages in Kattegat (Nordblom and Norell 2000, Raja-Halli et al. 2006). Isolates obtained from fish from the Black Sea belong to the subgroup Ie (Nishizawa et al. 2006, Altuntaş and Ogut 2010). VHSV genotypes II and III are

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found in wild marine fish in the Baltic Sea, the North Sea, and Skagerrak (Skall et al. 2005). VHSV-IV isolates, restricted to North America and Asia, have been collected from both freshwater and marine fish (Lumsden et al. 2007). Economically, the most essential genotypes appear to be Ia and IV, although most genotypes have not fully been investigated for their potential impact on wild and cultured species of fish.

Limited information is available on the virulence of the various genotypes of VHSV in European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), and gilthead sea bream *Sparus aurata* Linnaeus, 1758, which are used extensively in European aquaculture. Castric and de Kinkelin (1984) showed that genotype II, isolated from rainbow trout, developed clinical VHS in sea bass. Intraperitoneal injection of high doses of VHSV-II led up to 90% mortality, whereas challenge by immersion resulted in 50% mortality. Castric and Jeffroy (1991) subsequently showed that intraperitoneal injection of VHSV-II from elvers of the European eel, *Anguilla anguilla* (Linnaeus, 1758), was virulent to both sea bass and gilthead sea bream. However, the virulence of other genotypes of VHSV, e.g., genotype Ie common in Turkey, in sea bass and sea bream remains unknown.

In the Black Sea region of Turkey, VHSV-Ie is probably endemic (Altuntaş and Ogut 2010). VHSV was first isolated from rainbow trout in Georgia (Einer-Jensen et al. 2004). This genotype was subsequently isolated from turbot, *Scophthalmus maximus* (Linnaeus, 1758) (see Nishizawa et al. 2006) and whiting, *Merlangius merlangus* (Linnaeus, 1758) (see Altuntaş and Ogut 2010). Phylogenetic analyses indicated that the isolates from rainbow trout in Georgia and from whiting in Turkey were identical (Ogut unpublished). In another study, tests of virulence in three trout species showed that the Black Sea salmon, *Salmo labrax* Pallas, 1814, was the most susceptible to genotype Ie, whereas the levels of virulence were low in rainbow trout and brook trout, *Salvelinus fontinalis* (Mitchill, 1814) (see Ogut and Altuntas 2011). Nishizawa et al. (2006) reported that genotype Ie was also virulent to turbot. Increasing evidence indicates that the virulence of VHSV-Ie varies with host species, which is probable true for other genotypes of VHSV.

In this study, we surveyed the seasonal occurrence of VHSV-Ie in cultured sea bass and determined its virulence in sea bass and gilthead sea bream. This study examining potential impact of VHSV-Ie on sea bass juveniles at 12°C and 16°C may have strong implications for managing VHS, a notifiable disease, in the production of sea bass and gilthead sea bream.

MATERIALS AND METHODS

VHSV survey in cultured sea bass in cages. Sea bass juveniles (≈ 2 g), transferred from a hatchery in the Aegean Sea (Çamlı Inc., Izmir, Turkey) to a farm in the Black Sea, Yomra Bay (Dokabas Inc., Trabzon, Turkey), were surveyed monthly to determine the seasonal occurrence of VHSV. Sea bass ($n = 35$ per month) were sampled by nets

or divers from the same cage that was intentionally overstocked (100 000 fish per 14-m diameter cage) to enhance the occurrence of potential problems, e.g., viral disease occurrence. The samples collected monthly were transferred alive in aerated containers to the Fish Diseases Laboratory (Department of Fisheries Technology Engineering, Karadeniz Technical University) and processed virologically for viruses at the same day.

Challenge experiments. Two groups of sea bass juveniles, 0.243 ± 0.08 g and 0.34 ± 0.1 g, were obtained to assess the level of virulence of VHSV-Ie for juveniles of sea bass at 12°C and 16°C, respectively. The former challenge temperature was selected since it is the most proper temperature for the replication of VHSV (Ahne et al. 2002) and the latter was for the fact that most hatcheries of sea bass and sea bream have water supplies at around 16°C in the Mediterranean region. Gilthead sea bream (0.42 ± 0.06 g) were also challenged with VHSV genotype Ie. The fish of each species were brought from hatcheries, certified as viral-pathogen free, from the Aegean region of Turkey (Kılıç Holding, Mugla) and kept in a recirculating aquaculture systems (RAS) at the temperature of 16°C—the same as at the hatchery of origin. The water temperature was lowered by 1°C per day or two in the RAS until it reached 12°C, which was the temperature of seawater in the Black Sea at that time. The fish were then transferred to a flow-through system (12°C) for a week prior to the challenge for further acclimation to brackish water. Twenty juveniles of sea bass and gilthead sea bream were randomly sampled and tested for viral pathogens in five fish pools before initiation of the challenge experiments.

The origin and propagation of VHSV-Ie isolates in cell culture was previously described in Ogut and Altuntas (2010).

Sea bass. Sea bass juveniles were distributed in experimental groups of 40 fish per tank at 12°C experiment, or 30 fish per tank at 16°C experiment, into eleven 5-L tanks containing 3.5 L of water ($200 \text{ mL} \cdot \text{min}^{-1}$). Water inflow was stopped and volume was reduced to 1 L immediately before challenge. VHSV-Ie, propagated in BF-2 cells, were used at three challenge doses of VHSV-Ie as shown in Table 1. After 6 h of challenge with VHSV by immersion under static water with continuous aeration, water flow was resumed. Feeding, which was stopped one day prior to the challenge, was resumed the following day. Fish were observed for abnormal signs, and the mortalities were recorded and the dead fish were processed virologically daily during the exposure. The challenge experiments at 12°C continued for 29 days, whereas the one at 16°C was terminated nine days post infection with VHSV-Ie. On day 9 post infection at the 16°C challenge experiment, 15 fish from each tank were randomly sampled and individually tested by cell culture for the presence of VHSV to determine occurrence of virus in the absence of mortalities.

Gilthead sea bream. The same experimental design and similar tanks were also used for gilthead sea bream but with slightly different viral concentrations used for the

challenge (Table 1). The water temperature at the initiation of the experiment was 12°C, which gradually increased to 16°C by the end of the 16-day challenge experiment.

Tissue sampling for virology. In the surveillance study, tissue samples from kidney, liver, and spleen, pooled from five fish, were placed into serum-free Minimal Essential Medium with antibiotics (penicillin: 100 IU · mL⁻¹; streptomycin: 100 µg · mL⁻¹) and a fungicide (amphotericin-B: 0.25 µg · mL⁻¹) at a ratio of 1 : 10. After homogenisation, the samples were centrifuged for 20 min at 4000 g-force at 4°C and inoculated onto BF-2 (Wolf and Quimby 1976) and CHSE-214 (Fryer et al. 1965) cells in duplicate dilutions of 1 : 10 and 1 : 100. The cells showing cytopathic effects were inoculated once more, and positive samples were tested by ELISA (Test-Line Ltd Clinical Diagnostics, Brno, Czech Republic).

Parameters of water quality in both the surveillance and challenge experiments were monitored by a probe (YSI 556, Yellow Springs Instruments Inc., Yellow Springs, OH, USA). Water temperature, oxygen content, and pH at surface and depths of 5 m and 10 m, where sea bass in cages occupies, were monitored monthly.

Ethical issues. Fish were kept at optimum environmental conditions and cared according to the guidelines of Karadeniz Technical University Animal Care and Use committee. Both authors have the certificate for animal care and use given by the same committee.

RESULTS

VHSV surveillance of sea bass. VHSV, or any other viral pathogen that can grow on BF-2 or CHSE-214 cells, was not detected in sea bass samples during cage culture from

August to May in the Black Sea. Water temperature varied between 9.5°C and 27.4°C during the survey (Fig. 1).

Monthly percent increases in weight, that could be considered indicators of the health and welfare of sea bass, were highest in August and deteriorated gradually with the decline in water temperature, except for September when a sudden drop in weight gain was observed (Fig. 2). Observing an increased bacterial load on fish, a chloramine-T bath was applied in response to the decline in growth. The application led to an acceptable level of weight gain in the following month. Sea bass are not fed from January to mid-May when the water is cold (<13°C), and much weight loss (16%) was evident in April compared to December, by end of the active feeding period in the region. With the increase in ambient temperatures, fish began to gain weight in May, the beginning of the new grow-out period of sea bass and sea bream in the area.

Challenge experiments with sea bass. Moderate levels of disease-related mortality (DRM) was observed in the challenge experiment run at 12°C. Six log challenge with the VHSV-Ie generated from 17.5% to 25% VHS-related mortality (Table 1). There was DRM (up to 7.5%) at even the lowest dose, 10² TCID₅₀ virus per mL, tested. Higher ratios of prevalence (up to 33%) were observed in the survivor fish. All tanks challenged with various levels of virus presented infected survivors except the two replicates of the lowest doses tested. Moreover, the level of virus in mortalities were always above log(6), indicating HS progression in sea bass juveniles at 12°C (Fig. 3).

No mortality related to disease or virus replication was detected in the challenge experiment at 16°C. Three fish that died during the experiment were VHSV free.

Table 1

Results of the challenge experiment with VHSV-Ie immersion involving sea bass, *Dicentrarchus labrax* and gilt-head sea bream, *Sparus aurata*

<i>Dicentrarchus labrax</i> (16°C)						<i>Dicentrarchus labrax</i> (12°C)						<i>Sparus aurata</i> (12–16°C)					
AV	<i>n</i>	NM	DRM	DRM [%]	SI	AV	<i>n</i>	NM	DRM	DRM [%]	SI	AV	<i>n</i>	NM	DRM	DRM [%]	SI
6.0	30	0	0	0	0/15	6.3	40	0	10	25	3/15	6.1	20	0	1	5	0/20
6.0	30	1	0	0	0/15	6.3	40	0	8	20	5/15	6.1	20	0	3	15	0/20
6.0	30	0	0	0	0/15	6.3	40	1	7	17.5	3/15	6.1	20	0	3	15	0/20
4.0	30	0	0	0	0/15	5.3	39	1	4	10	3/15	4.1	20	0	3	15	0/20
4.0	30	0	0	0	0/15	5.3	40	1	3	7.5	2/15	4.1	20	0	1	5	0/20
4.0	30	0	0	0	0/15	5.3	39	14	5	12.5	3/20	4.1	20	0	0	0	0/20
2.0	30	0	0	0	0/15	4.3	40	0	0	0	0/15	2.1	20	1	0	0	0/20
2.0	30	1	0	0	0/15	4.3	40	0	3	7.5	0/15	2.1	20	0	1	5	0/20
2.0	30	0	0	0	0/15	4.3	40	1	2	5	1/15	2.1	20	0	0	0	0/20
Cont1	30	1	0	0	0/15	0	40	0	0	0	0/4/20	0	50	0	0	0	0/20
Cont2							40	0	0	0	0/4/20	0	50	2	0	0	0/20

Setup: 30 sea bass (at 16°C) and 40 (at 12°C) per tank, 20 gilthead sea bream, *Sparus aurata* per tank; exposure time: 6 h; 3 replicate tanks with 3 concentrations of VHSV-Ie; 20 surviving sea bass and gilthead sea bream were tested individually for the presence of virus; AV = amount of VHSV as Log(TCID₅₀) · mL⁻¹ used for challenge, *n* = number of fish per tank, NM = natural mortality, DRM = disease-related mortality, SI = survivors infected (20 surviving sea bass and sea bream were individually sampled and processed virologically).

Moreover, individual testing of 15 surviving fish in each treatment tank also showed that the virus was not present in the survivors after 9 days post infection.

Challenge experiments with gilthead sea bream. Mortality related to disease was observed in all three replicate tanks at the highest challenge dose. The highest cumulative percent mortality was 15% (Table 1, Fig. 4). No signs of disease were observed, and no viruses were present in the survivors of the challenge.

DISCUSSION

Sea bass transferred from hatcheries to the Black Sea were free of VHSV and stayed free of any viral pathogen for 10 months of their first year grow-out period even when temperatures in the Black Sea were appropriate ($\approx 12^\circ\text{C}$) for the replication of VHSV. VHSV-Ie caused moderate levels of virulence in sea bass at 12°C , and low levels of virulence in gilthead sea bream at $12\text{--}14^\circ\text{C}$. Especially VHSV-Ie infection in sea bass survivors was readily detectable one month post exposure.

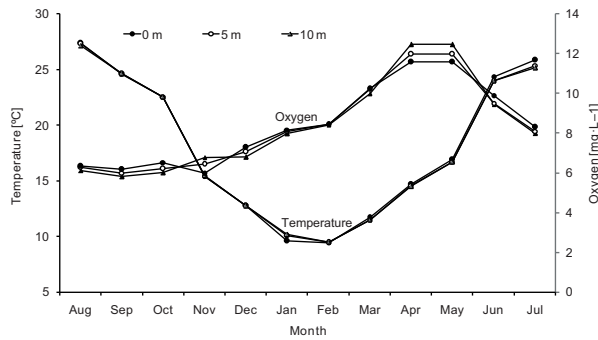


Fig. 1. Monthly fluctuation of temperature and oxygen content at the surface (0 m) and at depths of 5 m and 10 m during the survey in Yomra Bay ($40^\circ 57' 59.87''\text{N}$; $39^\circ 52' 43.57''\text{E}$)

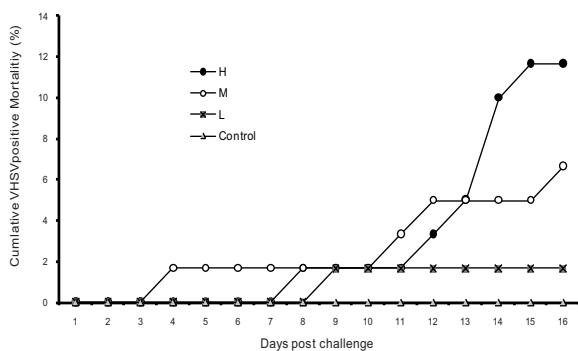


Fig. 3. Cumulative percent of VHS-related mortalities in fry of sea bass, *Dicentrarchus labrax*, bath-exposed to various doses of VHSV in triplicate at 12°C ; H = $10^{6.3} \text{TCID}_{50} \cdot \text{mL}^{-1}$, M = $10^{5.3} \text{TCID}_{50} \cdot \text{mL}^{-1}$, L = $10^{4.3} \text{TCID}_{50} \cdot \text{mL}^{-1}$, C = control; exposed to Minimal Essential Medium only; Titters (mean titters of daily mortalities) show the level of virus in recipient fish

Castric and de Kinkelin (1984) reported clinical VHS with high levels of mortality in sea bass challenged with genotype II (isolate 07-71) by both injection and immersion at 12°C . Challenge by injection produced a more severe disease (up to 50% mortality) than the challenge by immersion (20%) in 3 to 4 months old sea bass. We also observed similar rates of mortality (up to 25%) in the same size sea bass at the same temperature with VHSV-Ie. Similarly, sea bream were also susceptible to VHSV-Ie (15%). However, none of the sea bream survivors were tested positive for VHSV at 16°C , whereas up to 33% of sea bass exposed to VHSV-Ie about a month post exposure was tested positive for VHSV-Ie at 12°C . These results show temperature dependence of virus replication in vivo as reported by other researchers (Castric and de Kinkelin 1984, Skall et al. 2005). Moreover, the results also indicate that sea bass and sea bream are susceptible to potential natural outbreaks of VHSV-Ie in the region.

Higher densities of sea bass could potentially exacerbate infection transfer. In our study, the density of sea

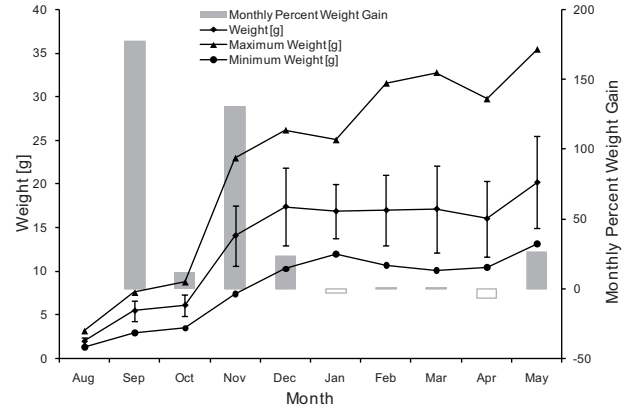


Fig. 2. Performance of sea bass, *Dicentrarchus labrax*, measured as weight, percent weight gain, and weight range (maximum and minimum), surveyed for viral pathogens of fish; Bars on the weight lines represent standard errors

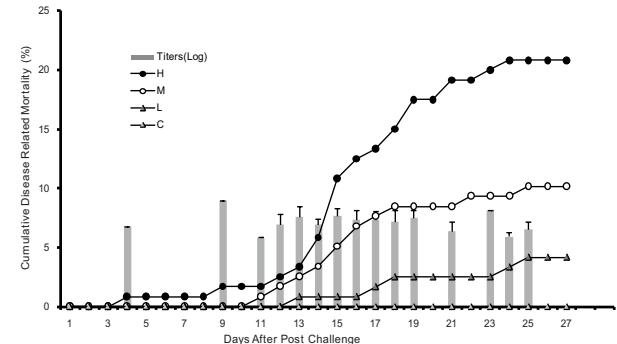


Fig. 4. Cumulative percent VHS-related mortalities in fry of gilthead sea bream, *Sparus aurata*, exposed to various doses of VHSV in triplicate at $12\text{--}14^\circ\text{C}$; H = $10^{6.1} \text{TCID}_{50} \cdot \text{mL}^{-1}$, M = $10^{4.1} \text{TCID}_{50} \cdot \text{mL}^{-1}$, L = $10^{2.1} \text{TCID}_{50} \cdot \text{mL}^{-1}$, C = control; exposed to Minimal Essential Medium only

bass was nearly four times higher ($5 \text{ kg} \cdot \text{m}^{-3}$) than the densities practiced in the hatcheries ($1.5 \text{ kg} \cdot \text{m}^{-3}$). The densities of gilthead sea bream were about the same as the densities in the hatcheries ($1.5 \text{ kg} \cdot \text{m}^{-3}$). Density thus seems not to be a factor in the occurrence and replication of VHSV in sea bass.

Some species native to the Black Sea region, e.g., Black Sea trout, are found susceptible to VHSV-Ie (Ogut and Altuntas 2011). The separation of cages keeping sea bass and Black Sea trout, e.g., by cages keeping sea bass, at the same farm may thus lessen the chance of transmission of VHSV-Ie between sea bass, sea bream and Black Sea trout at 12°C . Since many species of fish in the Black Sea are susceptible to VHSV-Ie (Ogut, unpublished data), monitoring sea bass and sea bream, if cultured together, for VHSV would also be an relevant tool for managing the transfer of virus between cultured to wild, wild to cultured, or cultured to cultured fish.

In this study, we intentionally challenged two-month-old sea bass under environmentally relevant temperatures (16°C) in the Aegean Sea region of Turkey and the temperatures that are known optimal (12°C) for the replication of VHSV. In Aegean region, the lowest water temperature in sea bass and sea bream hatcheries is about 16°C . In vivo replication of VHSV-Ie in sea bass at, or slightly below, 16°C is therefore a pertinent indicator of the magnitude of the risk posed by VHSV-Ie to the production of sea bass seed. These results strongly indicating no viral replication of VHSV-Ie at the temperature of 16°C in sea bass show that VHSV-Ie does not pose a risk to the production of sea bass seed in the region. The temperature of 16°C is very close to the reported upper limit for VHS occurrence. Goodwin and Merry (2011) identified an upper limit of 18°C for VHS intraperitoneal injection of VHSV-IVb. The upper limit for VHS caused by VHSV-I is 20°C (Isshiki et al. 2001). The absence of VHS related mortality and VHSV in survivors indicates either resistance of sea bass to VHSV-Ie or the absence of viral replication at 16°C . Moreover, the results also indicate that VHSV-Ie management in the Black Sea region necessitates not having hatcheries in the Black Sea region.

In conclusion, VHSV, or any other viral pathogen, was not detected in cultured sea bass in the Black Sea. Sea bass was resistant to VHSV-Ie at 16°C but displayed moderate level of susceptibility at 12°C . Sea bream presenting VHSV-related low levels of mortality around $12\text{--}14^\circ\text{C}$ were free of VHSV-Ie at 16°C . VSHV-Ie thus poses no risk to the production of sea bass seed in the Aegean region, where water temperatures are always at 16°C or above.

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