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ARTICLE

# A comparison of ectoparasite prevalence and occurrence of viral haemorrhagic septicemia virus (VHSV) in whiting *Merlangius merlangus euxinus*

Una comparación de la prevalencia de ectoparásitos y ocurrencia de virus septicémico hemorrágico viral (VHSV) en el merlán *Merlangius merlangus euxinus*

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**Resumen**.- Se examinaron 784 individuos del merlán *Merlangius merlangus euxinus* para su análisis virológico y parasitológico para establecer el efecto de la intensidad media de ectoparásitos sobre la ocurrencia del virus septicémico hemorrágico viral (VHSV) en peces del Mar Negro. El homogenizado de órganos internos (riñón, bazo e hígado) fue inoculado con células BF-2. Las células que mostraron efecto citopático (CPE) fueron probadas con ELISA para determinar la especie de virus. Las intensidades y prevalencia promedio de los ectoparásitos (*Trichodina* spp. y *Gyrodactylus alviga*) fueron determinadas sobre las branquias de los mismos peces. Los peces infectados con VHSV presentaron intensidades similares de *Trichodina* spp. (10,0 ± 1,8) a aquellos libres de VHSV (7,2 ± 1,0). Las intensidades medias de *Gyrodactylus alviga* fueron similares en los peces infectados con VHSV (1,8 ± 0,1) que en aquellos no infectados (3,3 ± 0,2). Estos resultados indican que los ectoparásitos no son los únicos responsables de la ocurrencia observada de VHSV. Otros factores, como el estrés del desove, fluctuaciones imprevistas en los parámetros de la calidad del agua por sí solo, o aditivamente con los ectoparásitos, pueden ser responsables de la ocurrencia de VHSV en el merlán.

**Palabras clave**: Merlán, septicemia hemorrágica viral, VHSV, *Trichodina* spp., *Gyrodactylus alviga*

**Abstract**.- Seven hundred and eighty-four whiting (*Merlangius merlangus euxinus*) were individually sampled for virological and parasitological examination to assess the effect of ectoparasite mean intensity on the occurrence of viral haemorrhagic septicemia virus (VHSV) in whiting in the Black Sea. The pooled homogenates of internal organs (kidney, spleen and liver) were inoculated onto BF–2 cells. The cells exhibiting cytopathic effect (CPE) were tested with ELISA to determine the virus species. The mean intensities and prevalence of ectoparasites (*Trichodina* spp. and *Gyrodactylus alviga*) were also determined on the gills of the same fish. The whiting infected with VHSV contained similar counts of *Trichodina* spp. (10.0 ± 1.8) than VHSV-free whiting (7.2 ± 1.0). The mean intensities of *Gyrodactylus alviga* were similar on the VHSV infected (1.8 ± 0.1) than uninfected fish (3.3 ± 0.2). The results indicate that ectoparasites were not alone responsible for the observed occurrence of VHSV. Other factors, such as spawning stress, sudden fluctuations in water quality parameters alone or additively to ectoparasites may be responsible for the occurrence of VHSV in whiting.

**Key words***:* Whiting, viral haemorrhagic septicemia, VHSV, *Trichodina* spp., *Gyrodactylus alviga*

## INTRODUCTION

Host-parasite interactions are highly complex and mostly involve more than one parasite in wild fish (Graham 2008). Disease severity, level of infectiousness, host availability, and fitness of host could all be altered by the impact of co-infection (Tompkins & Hudson 1999, Lello *et al*. 2005, Abu-Raddad *et al*. 2006, Cattadori *et al*. 2007).

Tremendous research effort has been spent on single infection cases in the same host especially in human diseases. Meantime, however, co-infections in the same host are drawing attention of researchers for the fact that host's immune response, disease severity, and duration of infection changes by the infection with a second parasite: HIV, malaria, and tuberculosis cases (Khan *et al.* 2001, Shivraj *et al.* 2006, Graham *et al.* 2007). Controlled experiments in the laboratory are necessary to reveal interactions of parasites on immune system as reviewed by (Behnke *et al*. 2001). That is, host parasite systems involving only few parasites are necessary to have a clear understanding of how parasites are interacting with susceptibility or any other host mechanism. On the other hand, as emphasized by Cattadori *et al*. (2007), field data especially on intra host dynamics are not trivial. The model host parasite system proposed to work here in the Black Sea brackish environment therefore may simulate both natural and laboratory environments to an extent due to the fact that there are only few parasite species living on whiting. It may help to provide insights for co-infections and their impacts on host-pathogen relationships, largely ignored in fish diseases literature.

Ogut & Palm (2005) reported that 89% of variation in parasite prevalence was explained by the variation in the rate of organic pollution in the surrounding environment. In their study, ectoparasite intensity was higher from January to April, coinciding with higher amount of organic pollution in the Black Sea. Telfer *et al.* (2010) reported large positive and negative effects of other infections. Other infections accounted for more variation explaining infection risks, than the variation related to environment or host factors. Interestingly, in a follow up study, Altuntas & Ogut (2010) isolated VHSV in the same period in whiting in the same region, indicating that ectoparasites and/or environmental organic pollution could seriously be stressing fish resulting in asymptomatic VHSV infections in whiting.

In this study, we assess the occurrence of VHSV in whiting infested with varying levels of the ectoparasite, *Trichodina* spp. This study may have insights in understanding impacts of protozoan parasites on the occurrence of viral fish pathogens in host-parasite systems involving carrier hosts.

## Materials and methods

### Sampling

Whiting *Merlangius merlangus euxinus* (Nordmann, 1840) were sampled using dip nets and hook and line at 2 locations: Yomra Bay (40°57’54.35"N, 39°51’38.22"E) and Surmene Bay (40°55’55.99"N, 40°12’21.44"E) off the coast of Trabzon Province from January through March in 2010 and 2011, during which VHSV is only present in the Black Sea ecosystem (Altuntas & Ogut 2010). To determine the number of trichodinids, uppermost left gill arc (Number 1) of each sampled fish was first removed aseptically and placed into formaldehyde (10%) containing gentamicin. Then, each fish was placed into a separate bag, transferred to the laboratory at 4°C in less than 2 h. After measuring length, weight, and determining sex of each fish, tissue samples from kidney, liver and spleen were collected aseptically, diluted 1:10, homogenized and centrifuged at 4000 g at 4°C. After further diluting to 1:100, each sample were inoculated onto BF-2 cell lines in duplicates. The inoculated cells were checked daily for the occurrence of cytopathic effect (CPE) and recorded for 14 days. All samples were reinoculated onto BF-2 cells regardless of CPE observation at 14 days postinoculation for blind passaging. The samples demonstrating CPE at the second passage were regarded as virus positive and each one of them were tested for viral haemorrhagic septicemia virus using a commercial ELISA (Test-Line, Clinical Diagnostics) as described using manufacturers recommendations with slight modifications as outlined by Altuntas & Ogut (2010). Opposite right uppermost gill arc was also sampled to determine the number of *Gyrodactylus* spp. The specimens were flattened, fixed and pictured (Leica LAS EZ 2.0.0.0) for the measurement of morphometric parts for species determination as outlined by (Malmberg 1970). Sodium dodesil sulphate (10%) was used after staining for easy observation of the structures.

Students t-test was used to compare square root transformed mean intensities of *Trichodina* spp. on 16 VHSV-infected and VHSV-free whiting. Sixteen VHSV-free whiting were randomly selected from 768 VHSV-free fish and their parasite load was compared to VHSV-infected fish. This procedure was repeated 10 times.

## Results

Two groups of ectoparasites; one protozoan (*Trichodina* spp.) and one monogenean, *Gyrodactylus alviga,* were encountered on the gills of whiting from January to March of 2010 and 2011 (Table 1). Of 784 fish sampled, 734 contained *Trichodina* spp. and 168 had *Gyrodactylus alviga* at varying levels (Table 2). Forty-eight fish, out of 784, had both ectoparasites while only 4 fish had *G. alviga* but no *Trichodina* spp.

In 2010 and 2011, the prevalence of *Trichodina* spp. on whiting (> 90%) were statistically similar in January, February and March ($\chi^2 = 0.78$; $P > 0.05$). Mean intensities were also statistically similar in 2010 ($\chi^2 = 2.35$; $P > 0.05$), but it sharply increased in March 2011 and it was significantly higher than the mean intensities in 2011 ($\chi^2 = 55.68$; $P < 0.05$). The prevalence of *Gyrodactylus alviga* increased gradually as its mean intensities decreased in 2010. Similar decreasing trend in the mean intensities of *G. alviga* was also observed in 2011, however, prevalence showed a reverse trend that of 2010.

**Table 1. Monthly prevalence and mean intensities of ectoparasites on whiting from June to March 2010 and 2011**/ Prevalencia mensual e intensidad promedio de ectoparásitos del merlán desde junio a marzo de 2010 y 2011

| | Time (Year/Month) | n | Weight (g) | Length (cm) | *Trichodina* spp. | | *Gyrodactylus alviga* | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Prevalence (%) | Mean Intensity | Prevalence (%) | Mean Intensity |
| 2010 | January | 152 | 20±8.9 | 13.7±1.9 | 96.0 | 50.5 | 7.2 | 9.9 |
| | February | 199 | 16.6±3.5 | 13.15±0.87 | 96.4 | 63.0 | 35.6 | 3.7 |
| | March | 93 | 16.7±3.8 | 13.2±0.9 | 91.3 | 49.7 | 44.0 | 2.0 |
| 2011 | January | 98 | 16.7±0.9 | 13.1±1.1 | 89.7 | 30.8 | 20.4 | 2.8 |
| | February | 105 | 16.5±3.6 | 13.2±1.0 | 97.1 | 96.7 | 11.4 | 1.5 |
| | March | 137 | 14.5±4.1 | 12.8±1.1 | 88.3 | 125.2 | 9.4 | 1.6 |

**Table 2. Morphometric measurements of *Gyrodactylus alviga*** / Medidas morfométricas de *Gyrodactylus alviga*

| Morphometric measurements / Sampling temperature | This study $12^0$C/18% | Dmitrieva & Dimitrov (2002) $13^0$C |
|---|---|---|
| Hook | | |
| Anchor length | 61.5±1.5 (59.7-63.7) | 64 |
| Anchor main part length | 40.9±1.3 (39.1-43.0) | 44 |
| Anchor point length | 22.9±0.8 (22.0-24.0) | 31 |
| Anchor root length | 21.5±2 (19.3-25.4) | 21 |
| Marginal hook | | |
| Marginal hook total length | 35.3±0.4 (34.6-35.8) | 34 |
| Marginal hook handle length | 28.3±0.2 (28.0-28.8) | 28 |
| Marginal hook sickle length | 7.3±0.2 (6.9-7.7) | 7 |
| Marginal hook upper point length | 3.0±0.3 (2.6-3.6) | - |
| Marginal hook lower point length | 2.6±0.5 (2.1-3.4) | - |
| Dorsal Bar | | |
| Dorsal bar length | 19.5±1.6 (16.2-21.0) | 21 |
| Dorsal bar width | 2.1±0.2 (1.7-2.6) | - |
| Ventral Bar | | |
| Ventral bar length | 23.82±1.1 (22.3-25.4) | - |
| Ventral bar width | 5.9±0.4 (5.3-6.5) | 6 |
| Ventral bar maximum length | 27.5±2.5 (29.3-32.7) | - |
| Ventral bar maximum width | 30.6±1.1 (40.9-48.9) | 31.5 |
| Ventral bar membrane length | 16.3±0.3 (16.0-16.9) | 22 |
| Antero-lateral process of ventral bar length | 5.3±0.7 (3.8-6.3) | 6 |
| n | 7 | - |

Low levels of mean intensity and prevalence of *Gyrodactylus alviga* were observed, not allowing for comparisons. Therefore, this organism was not included in the analyses as a factor.

### VHSV occurrence and spread

In 2010 and 2011, all the fish collected through January to March, were examined individually for parasitology, and checked for the presence of viral haemorrhagic septicemia Virus (VHSV) using cell culture. Only 16 fish, out of 784 individually sampled fish (overall 2% prevalence), tested positive for VHSV. Of the whiting tested positive for VHSV, 10 were female and 6 were male ($\chi^2$=1, $P > 0.05$) (Table 3).

Ten random sample pools were derived from the data by a random selection of 16 individual fish containing ectoparasites but not VHSV. The mean intensities (square

**Table 3. Ectoparasite presence and the characteristics of fish tested positive for viral haemorrhagic septicemia virus** / Presencia de ectoparásitos y las características de los peces positivos para el virus septisémico hemorrágico viral

| Time (Year/Month) | | Infected Fish | | | Ectoparasites | |
|---|---|---|---|---|---|---|
| | | Length (cm) | Weight (g) | F/M | *Trichodina* spp. | *Gyrodactylus alviga* |
| 2010 | January | 15.2 | 26.35 | F | 39 | 0 |
| | | 13.2 | 15.32 | M | 4 | 0 |
| | February | 14,1 | 18.68 | F | 16 | 0 |
| | | 13.3 | 17.10 | F | 201 | 1 |
| | | 13.5 | 17.42 | M | 17 | 1 |
| | | 12.9 | 14.00 | M | 134 | 2 |
| | | 13.7 | 18.22 | M | 20 | 0 |
| | | 14.4 | 22.72 | F | 28 | 0 |
| | | 12.6 | 16.08 | F | 13 | 1 |
| | | 14.5 | 23.62 | M | 315 | 5 |
| | March | 12.7 | 14.15 | F | 7 | 1 |
| 2011 | January | 13.3 | 18.15 | M | 105 | 0 |
| | March | 12.1 | 13.76 | F | 7 | 0 |
| | | 12.7 | 14.60 | F | 661 | 0 |
| | | 12.2 | 12.36 | F | 505 | 0 |
| | | 12.4 | 15.11 | F | 570 | 0 |

**Table 4. Statistical comparison (students' t-test on square root transformed data) of ectoparasite mean intensities on infected and uninfected whiting. Trichodinid loads of the 16 VHSV-infected whiting were compared to parasite loads of randomly selected 16 VHSV uninfected fish** / Comparaciones estadísticas (prueba t de student sobre los datos transformados con raíz cuadrada) de las intensidades promedio de ectoparásitos en merlanes infectados y no infectados. La carga de tricodínidos de los 16 peces infectados fueron comparados con las cargas parasitarias de 16 peces no infectados con VHSV seleccionados aleatoriamente

| Groups | *Trichodina* spp. Mean intensities (*) | | P-Value |
|---|---|---|---|
| | Infected | Uninfected | |
| 1. Group | 10.05 | 7.41 | 0.14 |
| 2. Group | 10.05 | 6.64 | 0.09 |
| 3. Group | 10.05 | 8.33 | 0.23 |
| 4. Group | 10.05 | 7.47 | 0.13 |
| 5. Group | 10.05 | 6.91 | 0.08 |
| 6. Group | 10.05 | 5.03 | 0.01 |
| 7. Group | 10.05 | 7.04 | 0.12 |
| 8. Group | 10.05 | 8.88 | 0.31 |
| 9. Group | 10.05 | 7.4 | 0.13 |
| 10. Group | 10.05 | 7.7 | 0.11 |

(*); Square root transformed

root transformed for normality) of each pool were compared to the mean parasite intensities (*Trichodina* spp.) of 16 fish infected with VHSV. The mean intensities of ectoparasites were always higher than that of uninfected group but not statistically significant except one. As shown in Table 4, the mean intensity of trichodinids was significantly higher in only one VHSV-infected group ($P < 0.05$).

## Discussion

The mean intensities of trichodinids present on VHSV infected whiting were always higher than on those fish not infected with VHSV, however, the differences were rarely statistically significant ($P < 0.05$). The data suggested that the load of *Trichodina* spp. on whiting had subtle effect on the occurrence of VHSV. Of the 10 randomly selected groups, a single group of VHSV-negative whiting contained significantly lower levels of parasite than VHSV infected whiting. Some other factors, *e.g.* spawning or water temperatures, alone or in combination to ectoparasite load may lead to higher prevalence of VHSV.

Variation in susceptibility of each host to a certain pathogen may not only be affected by the current exposure by a focal parasite but the presence and exposure history of second parasite (Boag *et al*. 2001). Consequently, we hypothesized that observed level and duration of Trichodinid exposure could lead to varying levels of susceptibility to VHSV in whiting. This approach may provide insights for 1) more frequent occurrence of VHSV in younger fish, 2) high variance in the counts of trichodinids in VHSV infected whiting. The trichodinids are always present on whiting at varying levels in the same system (Ogut & Palm 2005). Smaller fish are probably more vulnerable to the high exposure and first of this ectoparasite in their life cycle as Altuntas & Ogut (2010) reported that VHSV prevalence was higher in younger whiting. Bigger fish on the other hand may be more resistant to the potential negative effects of the parasite. All infected whiting had always-higher levels of Trichodinid counts comparing to the numbers of trichodinids on uninfected whiting. Acquired individual resistance to the negative effects of trichodinids could be responsible for the anticipated VHSV occurrence in whiting as was observed in some other animal diseases, *e.g.,* virus-nematode co-infections increases heterogeneities in susceptibility to certain diseases, having large impact on parasite transmission (Catadori *et al.* 2007).

Seasonal stressor such as water temperature fluctuations, spawning, nutritional deficiencies, and/or diseases may be important factors in VHSV outbreaks (Meier *et al*. 1994). VHS outbreaks usually occur at water temperatures below 15°C (Arkush *et al*. 2006). However, in our case, water temperature at depths where whiting live is always, including summer, below 13°C, optimal levels for the occurrence of VHSV, indicating that low water temperatures could not solely be responsible for the observed frequency of VHSV. Sudden fluctuations in water temperatures combined with high parasite load, however, could be responsible for the observed occurrence of VHSV in whiting.

The prevalence and mean intensities of *G. alviga*, the first report from the area, were lower during January to March, not allowing an assessment to the occurrence of VHSV. This would also mean that this ectoparasite alone has no influence on the occurrence of VHSV. Higher levels of monogeneans are usually observed on the same species of fish in warmer seasons (Gelnar 1987, Koskivaara *et al*. 1991). Therefore, this ectoparasite, constituting less variance than that of *Trichodina* spp., was not logical to use as a factor in assessing the occurrence of VHSV.

In brief, the occurrence of VHSV in whiting population in this study was about two percent, in line with the report of Altuntas & Ogut (2010) in the same region. The data suggested that parasite load was not the sole reason for the seasonal occurrence of VHSV in whiting in the Black Sea ecosystem but other factors as sudden chances in water quality parameters or spawning stress could alone or together with parasite load be responsible for the observed levels of VHSV prevalence in whiting.

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