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Parasites and endobiotic fungi in digestive gland cryosections of the mussel *Mytilus galloprovincialis* in the Northern Adriatic, Croatia

by

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Abstract

Histology has been used in the past to investigate the effects of diseases and parasite infections in native mussel populations that are often used as sentinel species in coastal environmental monitoring and as stock in mariculture. This paper presents the first study of parasite diversity using cryosections of the *Mytilus galloprovincialis* Lamarck, 1819 digestive gland. Mussels were sampled across the annual cycle at two sampling sites: St. Andrew and ACI Marina in the Northern Adriatic (Croatia). The protozoan of the genus *Nematopsis* Schneider, 1892 (Apicomplexa, Gregarina) was detected in digestive tubules, while the turbellarian (Urastomidae) *Urastoma cyprinae* von Graff, 1913 was found in the connective tissue at the edge of the digestive gland. The filamentous fungus *Alternaria* sp. (Fungi, Ascomycota) was detected in epithelial cells of the digestive tubule in cryosections. *Nematopsis* sp. occurred with the prevalence ranging from 20 to 100%, and the intensity of infection in less than 30 oocysts in most of the cases. *U. cyprinae* was detected in mussels sampled at St. Andrew and had a prevalence of 20% in September. Conidia of *Alternaria* sp. were found in mussels sampled at St. Andrew in September and November. Cryosections provide a useful and affordable means for monitoring parasites and endobiotic fungi.

Key words: mussel, *Mytilus galloprovincialis*, *Nematopsis*, *Urastoma cyprinae*, *Alternaria*, cryosection

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Introduction

Several parasites have been identified in bivalves from maricultured (Rayyan et al. 2004; Tuntiwaranuruk et al. 2008; Mladineo et al. 2012) and native mussel populations (Caceres-Martinez et al. 1998; Ceuta & Boehs 2012; Cova et al. 2015). There are several recent studies investigating the occurrence of protozoans from *Nematopsis* as the most common parasites of *Mytilus galloprovincialis* Lamarck, 1819 in Portugal (Francisco et al. 2010), Turkey (Özer & Güneydağ 2015a,b) and Ukraine (Gaevskaya 2006). The turbellarian *Urastoma cyprinae* von Graff, 1913 was observed in mussels from Portugal (Francisco et al. 2010), Turkey (Özer & Güneydağ 2015a) and Greece (Rayyan et al. 2004). Mladineo et al. (2012) described *U. cyprinae* associated with *M. galloprovincialis* in the Southern Adriatic Sea off Croatia.

Epibiotic and endobiotic fungi live on the surface and in the inner tissues or even in the cells of their hosts (Zhang et al. 2009). Filamentous fungi of the genus *Alternaria* have been found in bivalves (Zvereva & Vysotskaya 2005; Zvereva & Borzykh 2010; Borzykh & Zvereva 2015) and many other invertebrates (Zhang et al. 2009).

Mussels of the genus *Mytilus* are very important objects of marine environmental assessments and aquaculture industry. They are widely used in the food industry as well as in medicine, preventative health care and other commercial purposes (Venugopal 2008). *M. galloprovincialis* is the dominant mussel of the genus *Mytilus* (Hamer et al. 2012), at both intertidal and subtidal, sheltered and exposed sites. In Croatia, *M. galloprovincialis* has been maricultured extensively, particularly in the Northern Adriatic (Marušić et al. 2009). In this connection, studies of parasites and fungi inhabiting the internal organs of commercially important and cultivated mussels are necessary for successful development of mariculture and the safe use of mussels as food (Zvereva & Vysotskaya 2005). The study of parasites and pathogens from different mussel populations in response to regional differences is a subject of major interest, particularly in aquaculture where mussels originate from various sources (Bratoš et al. 2004; Pavičić-Hamer et al. 2016).

The objectives of this study were (a) to investigate the usefulness of cryosectioning in monitoring of mollusk infection and (b) to identify the range of parasites and fungi in mussels from two sampling sites in the Northern Adriatic, Croatia.

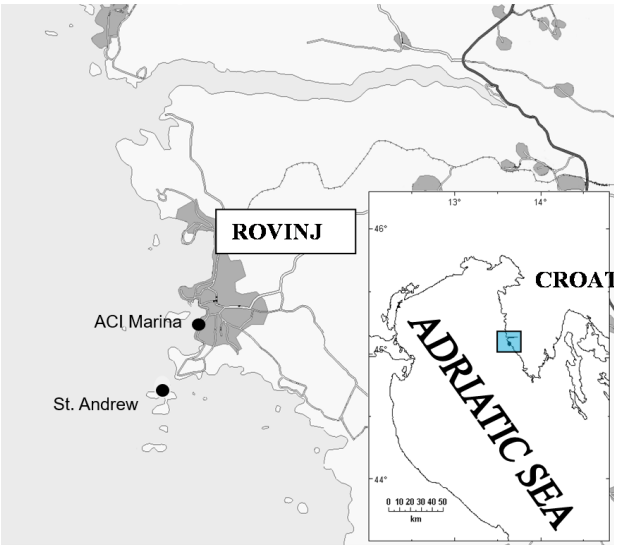

Figure 1

Geographical location of the sampling areas: (•) St. Andrew and ACI Marina, Northern Adriatic, Croatia

Materials and methods

Sampling sites and investigated bivalves

A total of 240 mussels were collected monthly (10 mussels from each of the two sampling stations) from September 2012 to August 2013 at two sampling stations in the Northern Adriatic Sea, Croatia (Fig. 1). St. Andrew is an island (45°03′31″N 13°37′28″E) located some 15 km from the urban and sewage outflow area (Kovačić et al. 2015). Adriatic Croatia International Marina (ACI Marina; 45°04′32″N 13°38′08″E) is located near a sewage outflow and boat processing area (Kovačić et al. 2016), which is characterized by an increased concentration of pollutants in sediments and biota comparable with nearby undisturbed environments (Bihari et al. 2004; Final report 2014). Both stations are located near Lim Bay, which is the largest mariculture area in Istria (Pavičić-Hamer et al. 2016) and one of the most important ones along the Croatian coast of the Adriatic (FAO 2015). The salinity and water temperature were measured *in situ* at both sampling locations with a pIONneer 65 apparatus (Radiometer Analytical S.A., France) during the sampling period. Seawater temperatures followed seasonal fluctuations and varied at the study sites between 9.7°C in March and 25.0°C in August (Table 1). Salinity varied during the study period, ranging from 29.2 PSU in May at station ACI Marina to 37.5 PSU in February at St. Andrew.

Ten mussels collected from each sampling station per month were immediately transported to the laboratory where digestive glands were removed. Digestive glands were placed in a straight

Table 1

Environmental factors: temperature and salinity measured at St. Andrew (SA) and ACI Marina (AM) sites

Month/ Environmental factor	Temperature (°C)		Salinity (PSU)	
Site	SA	AM	SA	AM
September	22.5	23.0	34.5	34.5
October	20.1	22.0	34.5	34.2
November	16.0	15.2	35.0	35.4
December	12.2	12.4	36.2	36.2
January	10.3	11.1	37.4	35.1
February	9.9	10.2	37.5	36.8
March	9.7	10.5	37.4	37.1
April	12.2	13.0	37.0	37.1
May	18.0	18.2	36.0	29.2
June	21.0	22.4	35.9	34.6
July	22.8	22.7	33.6	31.2
August	25.0	23.2	35.2	35.6

row across the aluminum cryostat chucks (five per chuck). Dissected mussel tissues were fixed with n-hexane, previously cooled in liquid nitrogen. Chucks were stored at -80°C until analysis. Before cryosectioning, samples were embedded in O.C.T.™ compound (Microm Inc. GmbH, Germany) and cut into 10 µm sections with a cryostat (Zeiss Hyrax C 50, Microm GmbH, Germany). Sections were stained with haematoxylin and eosin (Sigma-Aldrich, USA) and examined under a light microscope (Nikon, UK). All micrographs were captured using an Ikegami ICD-803P digital video camera and the Lim Screen Measurement™ Lucia G image capture system (Nikon, UK).

Following Saffo (1992), we have used the term "infection" in referring to all organisms, parasitically and endobiotically associated with its host. The prevalence and intensity of infection of each parasite was calculated according to Bush et al. (1997) as follows: prevalence as a number of infected individuals divided by the total number of individuals in a sample and expressed as a percentage; intensity as a number of parasites found in an infected mussel.

Statistical analysis

The relationship between the prevalence and the stations and sampling seasons was evaluated by the Chi-square (χ^2) test. The Spearman product-moment correlation was used to relate the infection to seawater temperature and salinity. All tests were performed with the Statistica 6.0. Software at a significance of $p < 0.05$.

Results

Mussel infection

Microscopic analysis of cryosections from the mussel digestive gland disclosed the protozoan *Nematopsis* (Fig. 2), the turbellarian *Urastoma cyprinae* (Fig. 3) and the fungus *Alternaria* sp. (Fig. 4).

Ungrouped oocysts of *Nematopsis* sp. were detected in most cryosections (Fig. 2A). Fig. 2B shows three oocysts located within the phagocyte in the connective tissue between digestive tubules of the mussel digestive gland. In some cryosections, five to eight oocysts were located within the phagocyte. The oocyst wall and the enclosed sporozoite were clearly observed in each oocyst (Fig. 2C). The parasitophorous vacuole that surrounded each oocyst located in the host phagocyte could also be observed. The prevalence of *Nematopsis* sp. was significantly higher at St. Andrew than at ACI Marina ($\chi^2 = 9.33$, df = 1, $p <$

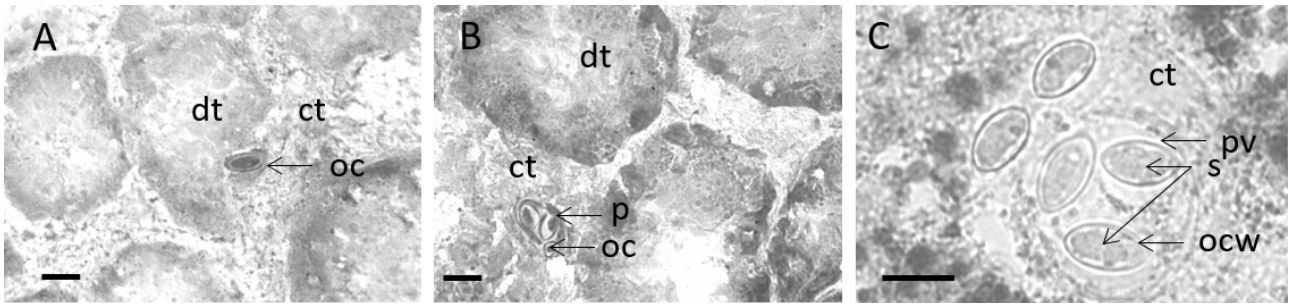

Figure 2

Light microscopy of transverse cryosections through the mussel *Mytilus galloprovincialis* digestive gland containing *Nematopsis* sp. A) One oocyst (oc) in connective tissue (ct) between digestive tubules (dt); B) host phagocyte (p) containing three oocysts (oc) in connective tissue (ct); C) oocysts located in a parasitophorous vacuole (pv) (lighter area). Note the oocyst wall (ocw) and the sporozoite (s) (scale bars = 10 µm)

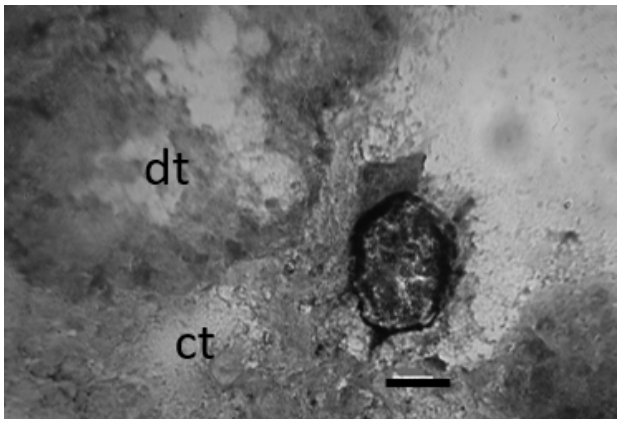

Figure 3

Light microscopy of *Urastoma cyprinae* located in connective tissue (ct) at the edge of the digestive gland and digestive tubules (dt) (scale bar = 20 µm)

0.05). Mussels sampled at St. Andrew had a prevalence ranging from 100% in September, November and August to 40% in July (Table 2). At ACI Marina, mussels were not infected in September, November, December, March and June. The prevalence in infected mussels from ACI Marina ranged from 20% in October to 60% in January and August (Table 2). The intensity of infection varied, with < 30 oocysts per section in most of the cases (maximum: 110 oocysts/mussel). We found a significant positive correlation between the infection intensity ($r = 0.854$, $p < 0.05$) and negative correlation with salinity ($r = -0.863$, $p < 0.05$) in mussels from St. Andrew station.

U. cyprinae was observed in mussels from St. Andrew in the connective tissue at the edge of the digestive gland (Fig. 3), with one specimen per mussel and the prevalence of 20% in September (Table 2). Mussels from ACI Marina were not infected with *U. cyprinae*.

The filamentous fungus *Alternaria* sp. was found in mussels sampled at St. Andrew in September and November (Fig. 4). Solitary conidia of *Alternaria* sp. were recorded in epithelial cells of digestive tubules in the mussel digestive gland. No apparent effect on epithelial cells was observed. We observed brown conidia, each with a short, cylindrical beak-like apical cell. The prevalence was 20% in September and 40% in November (Table 2). The intensity of infection was low (up to two conidia per section) and host reaction was not observed.

Discussion

Diseases and parasite infections in native and cultured mussel populations are usually assessed using paraffin-embedded tissue sections, which is a standard method for histological evaluation of parasite infections. In the present study, we have identified parasites and a filamentous fungus in the digestive gland of *M. galloprovincialis*, using cryosections. The preparation of cryosections does not involve the dehydration steps, typical of other sectioning methods, and thus the observation of specimens can usually be carried out in one day. Rapid freezing reduces the formation of ice crystals and minimizes the morphological damage. Frozen sections may be used

Table 2

Prevalence (P) and intensity of infection (I) with *Nematopsis* sp., *Urastoma cyprinae* and *Alternaria* sp. (range given in parentheses) from *Mytilus galloprovincialis* mussels sampled at St. Andrew (SA) and ACI Marina (AM)

Infection	*Nematopsis* sp. P (%) [I (range)]				*U. cyprinae* P (%) [I (range)]				*Alternaria* sp. P (%) [I (range)]			
Site	SA		AM		SA		AM		SA		AM	
September	100	[12 (2-27)]		-	20	[1]	-	-	20	[1]	-	-
October	80	[30 (12-38)]	20	[9 (9)]	-	-	-	-	-	-	-	-
November	100	[11 (6-17)]		-	-	-	-	-	40	[1]	-	-
December	60	[4 (2-5)]		-	-	-	-	-	-	-	-	-
January	80	[8 (1-17)]	60	[7(4-11)]	-	-	-	-	-	-	-	-
February	60	[6 (1-17)]	40	[6 (2-10)]	-	-	-	-	-	-	-	-
March	80	[9 (3-17)]		-	-	-	-	-	-	-	-	-
April	60	[12 (9-17)]	40	[1(1)]	-	-	-	-	-	-	-	-
May	60	[10 (6-15)]	40	[3(2-3)]	-	-	-	-	-	-	-	-
June	80	[13 (2-26)]		-	-	-	-	-	-	-	-	-
July	40	[74 (37-110)]	40	[3(1-4)]	-	-	-	-	-	-	-	-
August	100	[20 (2-60)]	60	[3(1-7)]	-	-	-	-	-	-	-	-

- not detected

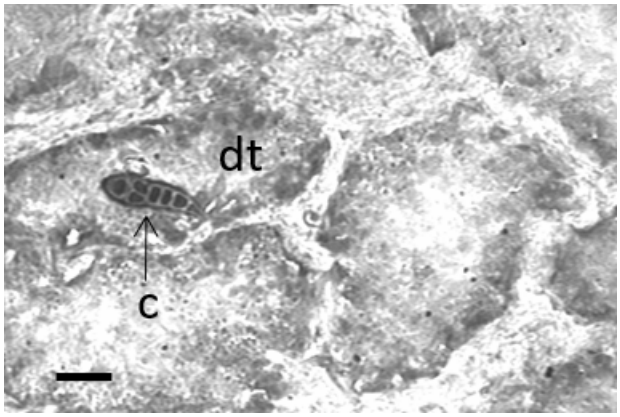

Figure 4

Light microscopy of transverse cryosections through the digestive gland of mussel *Mytilus galloprovincialis* containing conidia (c) of *Alternaria* sp. attached to epithelial cells of digestive tubules (dt) (scale bar = 20 µm)

for a variety of diagnoses such as immunohistochemistry, enzymatic detection, and in situ hybridization. This may be useful in mariculture where detection of parasites is needed to identify species in accordance with applicable legislation. In addition, cryosections offer the possibility of analyzing a number of other parameters in parallel, such as lipofuscin and unsaturated neutral lipid content (Brenner 2010; 2012), which is important in ecotoxicological studies of the marine environment. Therefore, we suggest the use of cryosections in the analysis of mussels, since this preserves the tissue as close to its natural state as possible. Moreover, in terms of quality, it is closely comparable to studies of formalin-fixed and paraffin-embedded sections (Tuntiwaranuruk et al. 2004; 2008; Francisco et al. 2010; Cova et al. 2015).

As evidenced by the study of Darriba et al. (2010), *Nematopsis* sp. is visible in histological examination as numerous dense oocysts. The prevalence for *Nematopsis* sp. reported in this study is higher in mussels than that reported at the Black Sea coast, at Sinop, Turkey (Özer & Güneydağ 2015a), but lower than the prevalence reported in oysters and mussels sampled at Bahia, Brazil (Ceuta & Boehs 2012; Cova et al. 2015). This discrepancy may be due to the differences in the geographical location and hosts. Mladineo (2008) also reported a high prevalence of *Nematopsis* sp. in the horse-bearded mussel *Modiolus barbatus* Linnaeus, 1758 in Mali Ston (Adriatic Sea, Croatia), as found in this study from the Northern Adriatic. Seasonal fluctuations in temperature and salinity throughout the year influenced the infection intensity of *Nematopsis* sp. It is possible that the increased seawater temperature in July caused the peak infection with *Nematopsis* sp. in mussels from St. Andrew, as it was reported in bivalves from the North-Western Adriatic Sea (Canestri-Trotti et al. 2000), the Gulf of Tailand (Tuntiwaranuruk et al. 2004) and the Aveiro Estuary in Portugal (Francisco et al. 2010). The observed pattern could be explained by a higher filtration rate in bivalves at higher temperatures (Bayne 1976). At a temperature of 20°C, the filtration rate immediately increases in response to an increase in seawater temperature. In addition, seawater salinity had an adverse effect on *Nematopsis* sp. infection intensity in mussels from St. Andrew, as observed in *Litopenaeus vannamei* Boone, 1931 (Jiménez et al. 2002). Significant differences were found between the prevalence in mussels from the two surveyed stations.

Tuntiwaranuruk et al. (2004) and Francisco et al. (2010) related infections of *Nematopsis* sp. to the habitat type and stated that heavy infections occurred in species living in a muddy substrate. The difference in the substrate found between two stations in Rovinj coastal area (Final report 2014) could support the above-mentioned theory; mussels from St. Andrew were more infected.

Low prevalence of the turbellarian *U. cyprinae* was found in our study, similarly as it was observed in *M. galloprovincialis* in Baja California, NW Mexico (Caceres-Martinez et al. 1998) and on the Black Sea coast at Sinop (Özer & Güneydağ 2015b). Moreover, low prevalence of *U. cyprinae* was found in the mangrove oyster *Crassostrea rhizophorae* Guilding, 1828 in the estuary of the Graciosa River in Taperoá, Bahia (Cova et al. 2015). In contrast, higher prevalence was found in *M. galloprovincialis* from the Southern Adriatic Sea, Croatia (Mladineo et al. 2012), Portugal (Francisco et al. 2010) and Greece (Rayyan et al. 2004). Several authors also reported that this parasite preferred autumn (Rayyan et al. 2004; Crespo-González et al. 2010) and was completely absent in winter (Özer & Güneydağ 2015b), just as it was observed in this study.

The results obtained in our study include the first record of *Alternaria* sp. in *M. galloprovincialis* in the Northern Adriatic (Croatia). Epibiotic and endobiotic fungi, like *Alternaria* sp., live on the surface and in the inner tissues of many invertebrates (sponges and coelenterates) and algae (Zhang et al. 2009). Since we found conidia inside the host cells, we could presume that it is an endobiotic fungus. Filamentous fungi of *Alternaria* sp. were associated with *Crenomytilus grayanus* (Bunker, 1853) and *Modiolus modiolus* (Linnaeus, 1758) (Zvereva & Vysotskaya 2005), Crassostrea *gigas* (Thunberg, 1793) (Borzykh & Zvereva 2010) and *Anadara broughtoni* (Schrenck, 1867)

(Borzykh & Zvereva 2015) from Peter the Great Bay, the Sea of Japan.

Additionally, research on parasites and diseases affecting mollusks of ecological and economic interest is important both for the management of natural stock and aquaculture (Boehs et al. 2010). *M. galloprovincialis* has been maricultured at five sampling stations in the Northern Adriatic (Pavičić-Hamer et al. 2016) near the sampling stations and general water currents could transport parasites preferentially northwards (Kovačić et al. 2016) into the mariculture areas of the Northern Adriatic.

In conclusion, we have determined that cryosections enable the diagnosis of parasite and fungi presence. *Nematopsis* sp. was a common parasite in the mussel digestive gland, followed by the turbellarian *U. cyprinae*. The results obtained in our study also indicate the first record of the filamentous fungus *Alternaria* sp. in *M. galloprovincialis* in the Northern Adriatic. Considering the high prevalence of gregarine *Nematopsis* sp. throughout the study period and *U. cyprinae* during a few months of the year, as well as fungi *Alternaria* sp., periodic monitoring of the health of this and other mollusks is recommended, particularly in mussels from mariculture areas near the study stations.

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