Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXIX

Settore Concorsuale di afferenza: 07/H3

Settore Scientifico disciplinare: VET/06

An integrated approach to improve the knowledge of Ostreid herpesvirus type 1 and the comprehension of mortality events in the Pacific oyster *Crassostrea gigas*

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Esame finale anno 2017

Collaborations

The present manuscript set out the results obtained thanks to a three-year collaboration between different research institutes, located in Italy and France. In particular, the main part of molecular analyses has been conducted at the LABÉO Frank Duncombe Laboratory, situated in Caen (Normandy – France), in the Microbiology-Virology Unit, where I have been hosted for 18 months. A huge contribution was also made by the Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta and especially by the Laboratorio di Ittiopatologia (Turin - Italy).

Acknowledgements

Ci sono essenzialmente quattro categorie di persone che vorrei ringraziare in modo particolare.

Le prime sono quelle che mi hanno sempre incorragiata a seguire le mie aspirazioni e che non hanno mai cercato di reprimere il mio gusto dell'avventura, qualunque fossero stati gli ostacoli e la difficoltà, insegnandomi fin dall'inizio che la cosa più preziosa che possedevo era la libertà di scegliere ogni giorno.

Le seconde sono sono state al mio fianco quando i dubbi comparivano e se dovessi fare nomi, Katia (che capisce bene di cosa parlo) e Tommy sareste i primi della lista.

Poi ci sono le persone che mi hanno regalato la loro fiducia, così, senza alcuna garanzia. Queste persone le voglio ringraziare poiché senza di loro non avrei potuto raggiungere questo traguardo: la Professoressa Marialetizia Fioravanti (questa volta dovrebbe essere l'ultima!!!), la Dottoressa Maryline Houssin (grazie a lei la Normandia sembra un posto quasi accogliente…) e il Dottore Marino Prearo (Doctor YES).

Vorrei ringraziare anche coloro che, al contrario, non hanno reso il compito facile, ma che hanno così contribuito, a loro modo, a non farmi dimenticare mai l'obiettivo.

E poi ci sono tutti gli altri:

gli allevatori che hanno partecipato al progetto: Alessandro e Francesca, Andrea, Cristian, Eugenio, Leonardo, Samuele e Stefano e il loro equipagi;

e i colleghi e amici: Guy, Elise, Lorena, Lucie, Marzia, Paola, Suzanne, Claudio, Gabriele, Enrico, Paolo e Vasco con i quali abbiamo a volte fatto le ore piccole giusto per qualche ostrica! Un giorno mi ringrazierete, nessuno saprà aprile le ostriche meglio di voi. Ah dimenticavo: piatta o concava?

E soprattutto c'è la mia Mamie, che mi fa sempre credere che sono la persona più importante del mondo.

Il y a principalement quatre types de personnes que je voudrais remercier d'une façon particulière.

Les premières sont celles qui m'ont toujours encouragée à suivre mes aspirations, sans jamais chercher à brimer mon gout pour l'aventure, quelles que soient les obstacles et les difficultés, m'apprenant depuis le début que la chose la più précieuse que nous possédons est la liberté de choisir chaque jour.

Les secondes sont celles qui ont été là quand l'ombre du doute pointait son nez. Katia (qui comprend bien de quoi je parle) et Tommy, vous etes les premiers sur la liste.

Il y a aussi les personnes qui m'ont donné en cadeau leur confiance, comme ça, sans garantie et sans qui rien n'aurait meme commencé: le Professeur Marialetizia Fioravanti (promis, cette thèse est la dernière!!!), le Docteur Maryline Houssin (qui fait presque sembler la Normandie un endroit accueillant…) e le Docteur Marino Prearo (Doctor YES).

Les dernier groupe comprend en revanche ceux qui m'ont rendu les choses plus difficiles mais qui ont contribué, eux aussi à leur manière, à ce que je n'oublie jamais mon objectif.

Et puis il y a tous les autres:

les éleveurs et leurs équipages: Alessandro et Francesca, Andrea, Cristian, Eugenio, Leonardo, Samuele e Stefano;

et les collègues et amis: Guy, Elise, Lorena, Lucie, Marzia, Paola, Suzanne, Claudio, Gabriele, Enrico, Paolo e Vasco avec lesquels on a veillé parfois jusqu'à tard juste pour quelques huitres! Un jour vous me remercierez, personne ne saura les ouvrir mieux que vous. Ah, j'oubliais: plate ou creuse?

Et surtout il y a ma Mamie, qui me fait toujours croire que je suis la personne la plus importante du monde.

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°C	degrees centigrade
µL	microlitres
AbHV-1	Abalone herpesvirus type 1
AMP	AntiMicrobial Peptide
ANOVA	ANalysis Of Variance
ARPAT	Agenzia Regionale per la Protezione Ambientale della Toscana
ARPAV	Agenzia Regionale per la Protezione Ambientale del Veneto
ASW	Artificial Sea Water
ATP	Adenosine TriPhosphate
AVG	Abalone Viral Ganglioneuritis
AVNV	Acute Viral Necrosis Virus
Bcl-2	B-cell lymphoma 2
BI-1	Bax Inhibitor 1
BIR	Baculoviral IAP Repeat
BIRP	Baculovirus Inhibitor of apoptosis Repeat Protein
BLAST	Basic Local Alignment Search Tool
BoHV-2	Bovine herpesvirus type 2
bp	base pairs
C. angulata	Portuguese oyster *Crassostrea angulata*
C. gigas	Pacific oyster *Crassostrea gigas*
CCV	Channel Catfish Virus
CFU	Colony Forming Unit
COI	Cytochrome c oxidase subunit I
cPCR	conventional Polymerase Chain Reaction
C_T	Cycle Threshold
d	days
DENV	DENgue Virus
DNA	DeoxyriboNucleic Acid
dUTP	deoxyUridine TriPhosphates
E	Early
EBV	Epstein-Barr Virus
EC	European Commission
EcSOD	Extracellular SuperOxide Dismutase
EFSA	European Food Safety Authority
EHV-1	Equine HerpesVirus type 1
EHV-2	Equine HerpesVirus type 2
EM	Electron Microscopy
EURL	European Reference Laboratory
FAO	Food and Agriculture Organization of the United Nations
fg	femtograms
FSC	Forward SCatter
g	grams
g	g-force
GNV	Gill Necrosis Virus
GU	Genomic Unit
H&E	Haematoxylin Eosin staining
HCMV	Human CytoMegaloVirus
HHV-8	Human HerpesVirus type 8

HIV	Haemocyte Infection Virus
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus
HVEM	HerpesVirus Entry Mediator
IAP	Inhibitor of Apoptosis
IC	Internal Control
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate Early
IFN	InterFeroN
IHC	ImmunoHistoChemistry
IPNV	Infectious Pancreatic Necrosis Virus
IR_L	Internal Repeat Long
IR_S	Internal Repeat Short
ISG	Interferon Stimulated Gene
ISH	In Situ Hybridisation
IκB	Nuclear Factor-Kappa B Inhibitor
La	Late
L	Litres
LPS	LipoPolySaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time Of Life
mg	Milligrams
min	Minutes
MJC	MaJor Capsid protein
mL	Millilitres
MLSA	Multi Locus Sequence Analysis
mm	Millimetres
mtDNA	Mitochondrial DNA
N	Sample Size
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κB	Nuclear Factor-Kappa B
NGS	Next Generation Sequencing
NM	Nuclear Marker
nm	Nanometres
OIE	Office International des Epizooties
ORF	Open Reading Frame
OsHV-1	Ostreid herpesvirus type 1
OsHV-1-SB	Ostreid herpesvirus type 1 *Scapharca broughtonii* strain
OVVD	Oyster Velar Virus Disease
PCR	Polymerase Chain Reaction
Poly(I:C)	Polyinosinic: polycytidylic acid
qPCR	quantitative PCR
REPAMO	REseau de PAthologie des MOlluques
RER	Rough Endoplasmic Reticulum
RFLP	Restriction Fragment Length Polymorphism
RING	Really Interesting New Gene
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
rtPCR	real-time PCR
s	Second
SBS	Sequencing By Synthesis
SSC	Side SCatter

SuHV-1	Suis HerpesVirus type 1
TEM	Transmission Electron Microscopy
TGN	Trans Golgi Network
THV	Tupaia HerpesVirus
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAIL	Tumor-necrosis-factor Related Apoptosis Inducing Ligand
TR_L	Terminal Repeat Long
TR_S	Terminal Repeat Short
U_L	Long Unique region
U_S	Short Unique region
UV	UltraViolet
VZV	Varicella Zoster Virus
WHO	World Health Organization

INTRODUCTION

Introduction

Consumption of fish, defined as finfish (vertebrates) and shellfish (invertebrates), provides energy, protein and a range of other important nutrients, with beneficial health outcomes for humans (FAO/WHO, 2010; EFSA, 2014). Moreover, eating fish is part of the cultural traditions of many people, in several populations around the world. Thanks to aquaculture activities, 73 million tons of fish are produced every year in the world, 89% of which by the Asian continent (FAO, 2014). It is interesting to highlight that 22% of this production consist in shellfish, oysters in particular, with a production of 5 million tons by year. The genus *Crassostrea* represents 99% of this production. Unlike other forms of aquaculture and thanks to their filter-feeding habits, bivalve farming can be considered as a sustainable animal-derived protein source.

In Europe, with 631,800 t per year, the bivalve molluscs represent 21% of the total aquaculture production. Essentially, these bivalves belong to few genera or species: mussels (*Mytilus* spp.) rank first with 495,000 tons produced in 2014, far forward oysters (*Crassostrea* spp.) with 89,000 t/year, and clams, *Ruditapes philippinarum* (Adams & Reeve, 1850) with 32,000 t/year (FAO, 2014). The European flat oyster, *Ostrea edulis* (Linnaeus, 1758) and the European carpet shell, *Ruditapes decussatus* (Linnaeus, 1758) represent only 3% of the oyster production and 10% of the clam production respectively. Italy ranks third among bivalve mollusc producing countries in Europe with 110.645 t/year (FAO, 2014). Though clams and mussels still account for the bulk of national production with 31.600 t/year and 79.000 t/year respectively (FAO, 2014), the Pacific cupped oyster, *Crassostrea gigas* (Thunberg, 1793) is becoming an increasingly important product, even if it represents today less than 1% of the total bivalve production and concerns mainly three areas, Sardinia and, to a lesser extent, Liguria, and the Po Delta. In the past, in Italy, the culture of the native flat oyster *O. edulis* has been practised, since antiquity to the end of the 19th century, before of being almost completely abandoned nowadays. During the late sixties, several experimental trials of cupped oyster farming were conducted along the Italian coasts, through the introduction of both *C. gigas* and *Crassostrea angulata* (Lamarck, 1819), but the real producing activities started about ten years ago. However, it is difficult to establish if some *Crassostrea* spp. wild populations, especially in the Northern Adriatic, were not yet present, may be introduced inadvertently long time ago by shipping from their native area in East Asia. In any case, finding optimal environmental conditions, cupped oysters successfully established and spread,

and today wild populations are intensively present along the Northern and Eastern Adriatic coasts. France remains the main cupped oyster producer in Europe with 76,000 t produced in 2014 (FAO, 2014).

In Europe, the farming technique varies among the regions, in order to fit to the different environmental conditions. Intertidal oyster cultivation is the most significant technique practised in France along the Atlantic and Channel coasts, but in Mediterranean Sea, where the tidal range is restrained, other techniques must be used such as gluing oysters onto ropes, in the “Etang de Thau” (France), farming them in lantern-nets or baskets, or using floating bags in the “Stagno di San Teodoro” (Italy). The seed supplying is achieved by two ways: natural collection on the wild, mainly practised in the Arcachon Bay and in Charente-Maritime (France) and hatchery production. This second source is not dependant on annual fluctuations, it allows procurement over a longer period of the year, it provides also triploid individuals, batches of homogeneous size, a low rate of individuals attached to each other, and the possibility of genetic selection. Actually, no commercial hatchery for *C. gigas* is present in Italy and the spat collection in open sea is not performed, compelling the producers to import spat from France. Currently, the farming cycle is about three years but may be shorter in the Mediterranean waters. The progresses accomplished in the zootechnical field had influenced positively the intensification of farming practices and a specialization of some coastal areas in oyster culture. However, the high stocking density increases host contact rates and stress, and reduces water quality making aquaculture susceptible to disease outbreaks. Moreover, the common use of bivalve stock transfers between productive areas, also situated in different countries, improves the risk of the insurgence and diffusion of infective diseases.

The rearing cycle, from spat to commercial product, takes place in an environment scarcely controllable and the oyster farming, during its history, has always been characterised by fluctuations of the production. Livestocks are subjected to natural environmental conditions that may compromise their growth and survival. In this scenario, infectious diseases have a heavy impact in marine aquaculture, with important economic consequences (Lafferty *et al.*, 2015). For instance, a report titled “Procédures de couverture des risques conchylicoles”, delivered by the “Conseil général de l’agriculture, de l’alimentation et des espaces ruraux”, “Conseil général de l’environnement et du développement durable”, and “Inspection générale des affaires maritimes” jointly, appointed by the French Ministry for Food, Agriculture and Fisheries and Ministry for Ecology, Energy, Sustainable Development

and Sea, established at 172 million euros the amount of damages caused by summer mortalities of Pacific oysters only for the year 2008. Besides, contrary to what happens in finfish aquaculture, chemotherapy and vaccination are not suitable solutions in the case of mollusc disease control. In the past, the oyster industry has been periodically affected by various infectious diseases caused by parasites, viruses, or bacteria. In France, the European flat oyster production has fallen from 20,000 tons in the first seventies to 2,300 tons in 1985 (Grizel, 1985; Goulletquer and Heral, 1997) because of the insurgence of two parasitic diseases: marteiliosis in 1968, and bonamiasis in 1979, due to the protozoans *Marteilia refringens* (Comps, 1970a; Herrbach, 1971, Grizel *et al*., 1974) and *Bonamia* spp. (Pichot *et al.*, 1980, Comps *et al.*, 1980, Comps, 1983). Nearly in the same period, farmed and wild populations of Portuguese cupped oyster *C. angulata* were practically decimated in few years by an irido-like virus (Comps *et al.*, 1976). This event has been responsible of the introduction of another species of cupped oyster, the Pacific oyster *C. gigas*, to guarantee the prosecution of farming activities. Regrettably, since April-May 2008, juvenile stages of Pacific oyster, in turn, have been affected by dramatic mortality events in all the French producing regions, with mortality rates ranging between 60% and 100% (Cochennec-Laureau *et al*., 2009; Renault *et al*., 2009). These events were associated with a new variant of the Ostreid herpesvirus type 1 (OsHV-1) termed µVar (Segarra *et al*., 2010) and since them, they were observed in several regions of the world, always correlated with the presence of OsHV-1 microvariants (OIE, 2014). In addition, commercial size oysters are affected by anomalous mortalities since 2012 in France, associated with *Vibrio* infections, and even if the disease course is less acute than in the case of young oysters, these events represent a significant threat to the oyster industry for the huge economic losses they generate.

Given the extent of these phenomena, and the increasing alarm of farmers, international cooperation is highly desirable, as expressed during the Annual Meeting of National Reference Laboratories for Molluscs Diseases in Rochefort on the 18th March 2013. Joint operations could accelerate the comprehension of these events and the finding of some solutions. A priority must be given to the study of OsHV-1. In fact, despite the heavy economic impact of virus-induced mortalities, international effective measures to prevent and control the disease are nowadays unavailable, in part because of the scarce information on the real diffusion of the virus and its variants, and their relative effective pathogenicity. Furthermore, some important questions emerge and, to try to answer them, a team work, made up of Italian and French Research Institutes was realised, with the final aim to give some solid

elements for future risk assessment activities, for the prevention of spread, and to limit economic losses that farmers have to face.

The main fields were:

- What is the real diffusion of OsHV-1 in the wild? Considering the close contact between wild and reared oyster populations and the excellent water capacities as vehicle of infectious agents, it is important to gather information on the health status of natural bivalve populations related to OsHV-1, in order to assess the potential risk of their contamination by infected farmed individuals or, on the contrary, to establish if they may act as virus reservoir.
- What is the genetic diversity of OsHV-1, especially in the wild? What are the evolutionary relationships among the genotypes?
- It has been postulated that the virulence of OsHV-1 microvariants is higher but what is the origin of this virulence if compared to the reference genotype, present before 2008? What are the putative virulence factors?
- What are the other factors that influence the OsHV-1 capacity of infection and pathogenicity? Even if a new variant was associated with the recrudescence of mortality events in young oysters in 2008, it is assumed that other factors may play an important role in the intensification of these events, such as environmental factors, synergy of the pathogenic effects during co-infections, and host factors such as physiological state (Baud *et al*., 2012).

In a period of expansion of oysters farming in Italy, the present work aimed to contribute to this challenge, approaching several important aspects connected with oyster health management in Italy. The information collected during the present study will aslo contribute to improving the global knowledge on oyster pathology.

The first part of the manuscript, called “Literature Review”, gathers information on the Pacific oyster *C. gigas* and on its pathogens, OsHV-1 in particular.
The second part discusses the results of the experimental activities and is partitioned in four chapters. The first chapter is dedicated to the improvement of knowledge of the health status of wild populations of molluscs related to OsHV-1. This concerns also investigations on genetic aspects of the viral specimens isolated in field and the identification of the species of

cupped oysters present in Italy, through the development of a molecular tool. The second chapter relates the sequencing of the whole genome of OsHV-1 µVar. The third chapter details the results of the multi-site tests: in order to evaluate the possible effects of different factors on the insurgence of anomalous mortality events, oyster pools, from the same batches, were placed in several sites characterised by different environmental conditions, in Italy and France. The effect of ploidy, age of oysters, and allocation date on mortality rate, prevalence and load of OsHV-1 and *Vibrio aestuarianus* were tested, along with the effect of environmental parameters, and presence of potentially pathogenic microorganisms. Finally, in the fourth chapter we described two mortality events occurred in farmed stocks: the first affecting adult individuals in San Teodoro (Italy) and associated with bacterial pathogens, and the second occurred in Normandy and affecting spat during June 2016.

PART I
LITERATURE REVIEW

1.1. The host: the Pacific oyster *Crassostrea gigas* (Thunberg, 1793)

1.1.1. Taxonomy and species distribution

The Pacific oyster *C. gigas*, also called Japanese oyster, is a marine bivalve mollusc, member of the *Ostreidae* family. Its exact classification (Marshall and Gofas, 2015) is reported in Table 1.

Kingdom	*Animalia*
Phylum	*Mollusca*
Class	*Bivalvia*
Subclass	*Pteriomorphia*
Order	*Ostreida*
Superfamily	*Ostreoidea*
Family	*Ostreidae*
Subfamily	*Crassostreinae*
Genus	*Crassostrea*
Species	*Crassostrea gigas*

Table 1 Taxonomic classification of the Pacific oyster *Crassostrea gigas.*

To date, the genus *Crassostrea* includes 22 species (Bouchet and Gofas, 2012):

Crassostrea aequatorialis (d'Orbigny, 1846)

Crassostrea angulata (Lamarck, 1819)

Crassostrea ariakensis (Fujita, 1913)

Crassostrea belcheri (G. B. Sowerby II, 1871)

Crassostrea bilineata (Röding, 1798)

Crassostrea brasiliana (Lamarck, 1819)

Crassostrea columbiensis (Hanley, 1846)

Crassostrea corteziensis (Hertlein, 1951)

Crassostrea cuttackensis (Newton & Smith, 1912)

Crassostrea dactylena (Iredale, 1939)

*Crassostrea dianbaiens*is (Xia, Wu, Xiao & Yu, 2014)

Crassostrea gigas (Thunberg, 1793)

Crassostrea hongkongensis (Lam & Morton, 200)

Crassostrea ingens (Zittel, 1865)
Crassostrea mangle (Amaral & Simone, 2014)
Crassostrea nippona (Seki, 1934)
Crassostrea praia (Ihering, 1907)
Crassostrea rhizophorae (Guilding, 1828)
Crassostrea rivularis (Gould, 1861)
Crassostrea sikamea (Amemiya, 1928)
Crassostrea tulipa (Lamarck, 1819)
Crassostrea virginica (Gmelin, 1791)

The species *Crassostrea laperousii* (Schrenk, 1861), *Crassostrea posjetica* (Razin, 1934) and *Crassostrea talienwhanensis* (Crosse, 1862) are considered as synonyms of *C. gigas*. In contrast, even if there is evidence of viable interspecific hybridisation between *C. gigas* and the Portuguese oyster *C. angulata* (Gaffney and Allen, 1993; Huvet *et al*., 2001, 2002, 2004) and after numerous controversies, the two species are nowadays considered as distinct species. However, the phenotypic characters are not sufficient to discriminate the two species. Several genetic studies, based on mitochondrial DNA (Boudry *et al.*, 1998; O'Foighil *et al*., 1998) and microsatellite data (Huvet *et al*., 2000a, 2000b) provided evidence that the two taxa are genetically distinct although closely related (Batista *et al*., 2005). In particular, the estimation on nucleotide divergence (5.26%) of the cytochrome c oxidase subunit I gene (*COI*) sequence suggests that populations of *C. gigas* and *C. angulata* are closely related and may have diverged only one to two million years ago (O'Foighil *et al*., 1998). Studies involving microsatellite markers also confirmed that there are low but clear genetic differences between the two taxons. Before the intentional introduction of *C. gigas* from Japan to France during the early seventies, to face the mass mortality events that wiped out the Portuguese oyster from French coasts, the two populations were believed to have a well separated geographical distribution: *C. angulata* on the European Atlantic seaboard (that explains the common name "Portuguese oyster") and *C. gigas* in Asia. However, from the recent phylogenetical studies, it seems clear that the European *C. angulata* populations were introduced in the XVI or XVIIth century from Taiwan, firstly in Portugal before their spread northward. Nevertheless, the relationship between the two taxa in overlapping home range locations is not clear. Actually, *C. angulata* and *C. gigas* are listed as separate but very closely related species and they still potentially conspecifics. Their global geographical distribution is represented indiscriminately on Figure 1.

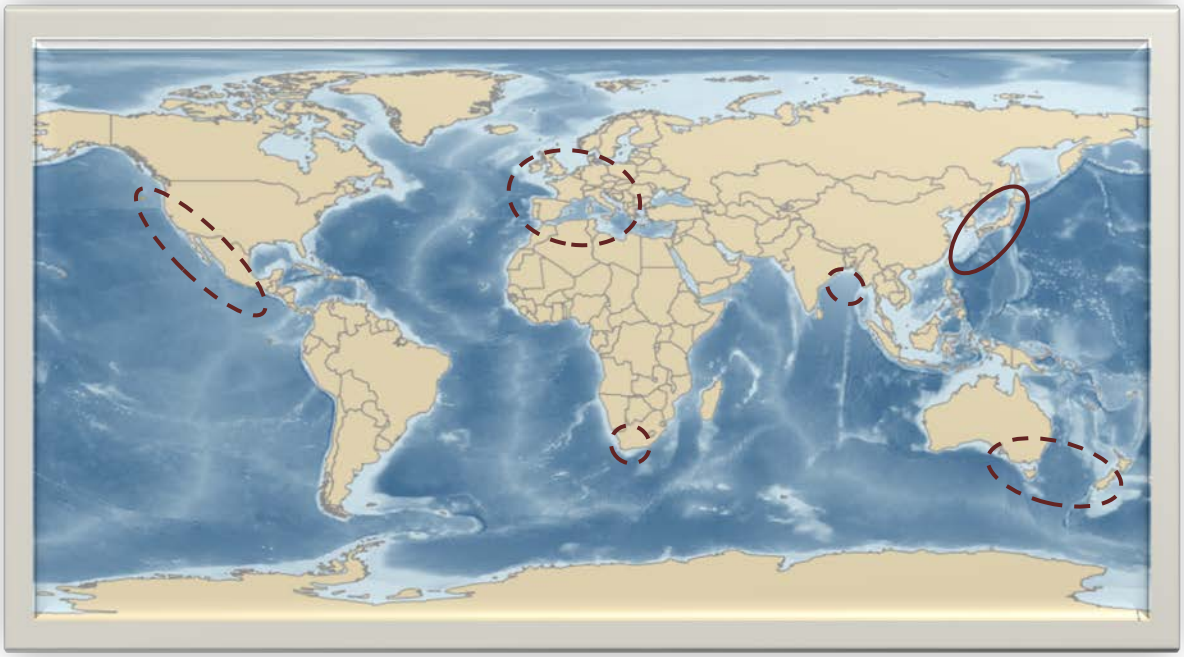


Fig. 1 Geographical distribution of *C. gigas*/*C. angulata*
Continuous line: native area; dotted line: introduced populations (adapted from OBIS ver. 1.0)

1.1.2. Habitat

Natural Pacific oyster populations are usually established in sheltered areas along the coasts, in the intertidal zone or until a depth of about ten meters, preferably in estuarine or lagoonal environments. In fact, these ecosystems characterised by high trophic levels resulting from the mixing of inland waters, rich in nutrients, with marine waters, are suitable for filter-feeding species. As an intertidal species, it is very tolerant to various abiotic conditions. *C. gigas* is a euryhaline organism, able to adapt to a wide range of salinities. Both optimal and tolerance ranges for growth and spawning are very large, as reported in Table 2. The species is also extremely resistant to environmental stress from high metal exposure (Rainbow and Phillips, 1993; Boening, 1999; Funes *et al*., 2006). In the areas where wild beds develop, they tend to constitute reefs that provide a shelter for other marine species, even if the invasive character of *C. gigas* is recognised (PROGIG, 2009).

Temperature (°C)		**Salinity (ppt)**	
Growth	Spawning	Growth	Spawning
3-35 (11-34)	16-35 (20-25)	10-42 (35)	10-30 (20-30)

Table 2 Temperature and salinity ranges for adult *C. gigas*.
Optimal ranges in parentheses (Mann *et al*., 1991)

1.1.3. Anatomy

Mollusca is one of the largest and diverse animal phylum, second only to *Arthropoda*, with over 46,000 described marine species, allocated in seven major classes, with a soft body as common character. In the case of bivalves, soft tissues are protected by a hard shell consisting in two valves, held together by a horny ligament. *Ostreidae* valves are asymmetrical. The left valve of *C. gigas* is larger and deeply cupped (Figure 2). It is always the left valve to be cemented to the substrate, guarantying the sessile life to this mollusc. The external shell colouring varies, on the basis of the geographical origin, from off-white, brownish, to deep purple. The inside of the shell is pearly-white with a single large muscle scar. As observed for the colour, the shape is also fashioned by the influence of both environmental and genetic factors. Along the European coasts, *C. gigas* and *O. edulis* populations can share the same habitat. However, the two species are easily distinguishable: the left valve in *O. edulis* is less cupped, the shape is rounder, the junction line between the two valves is not curled and the presence of a dozen lateral hinge teeth is only observed in the genus *Ostrea* (personal observation).

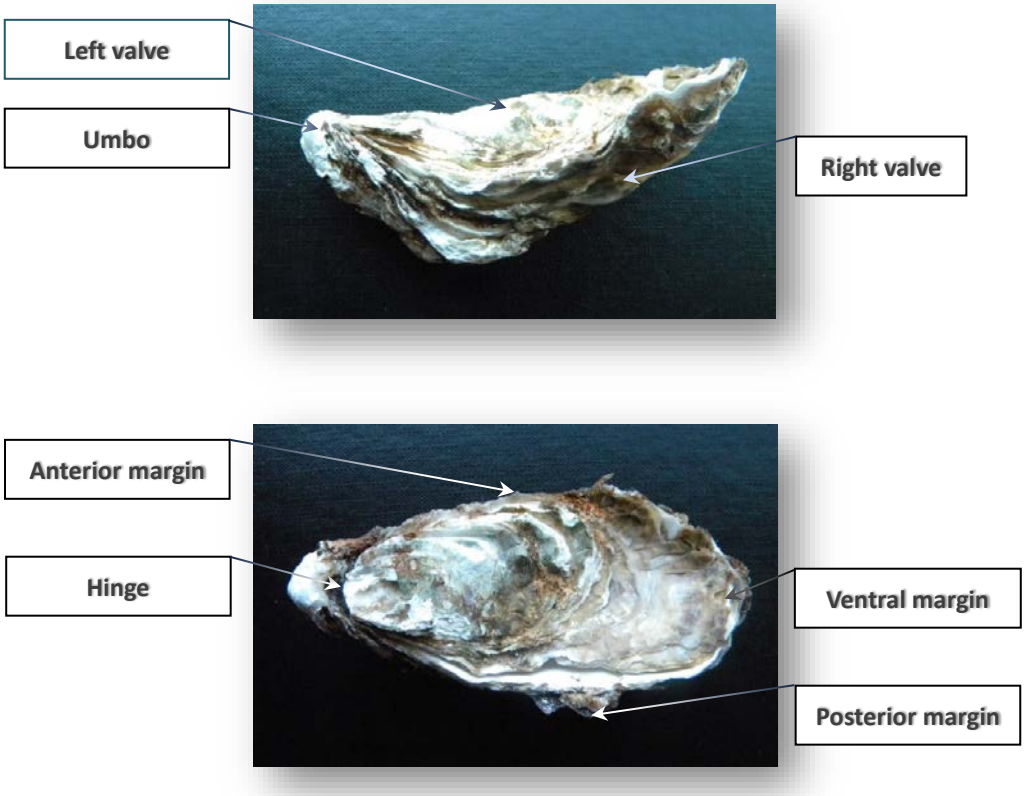


Fig. 2 External shell features of *C. gigas* (E. Burioli)

Molluscs are metazoan triploblastic coelomates, even if the coelom is reduced just to the cavities surrounding the gonads, the heart and the excretory organs.

The mantle, also called pallium, is an extension of the dorsal tegument that hangs down, forming two lobes around the body that constitute the mantle cavity (Figure 3). It is composed of connective tissue surrounding muscle fibers, haemolymphatic vessels, nerves, and is covered by a single layer of epithelium. The two lobes are fused in the cephalic region and form a cap that protects the mouth and the ciliated labial palps. The edges of two halves of the mantle have three folds and the medial fold shows very short tentacles with numerous sensorial organs. The mantle cavity protects the gills and the excretory, anal and genital orifices. This chamber serves for the ingress of water and nutrients and for the expulsion of the excreta and genital products. Important physiological functions are performed by the mantle, such as the production of the shell and the energy storing, in the form of glycogen that is the primary energy store in bivalves (Gabbott, 1983). The mantle epithelium includes numerous glandular cells producing mucus with protective functions and acting as a barrier against external agents.

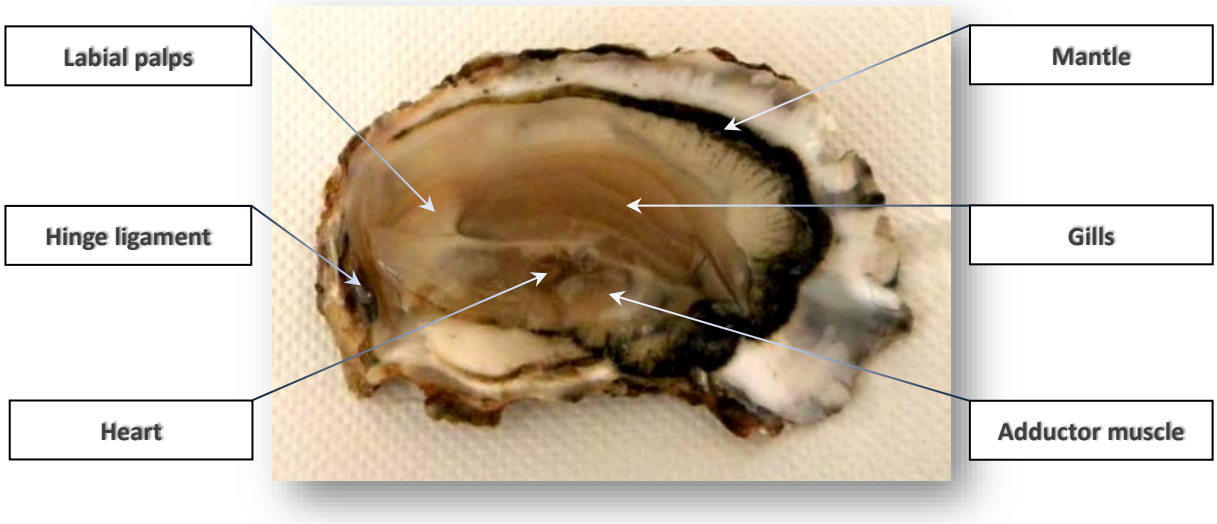


Fig. 3 General anatomy of *C. gigas* after the removal of right valve (E. Burioli)

In the *Ostreidae* family, the foot is atrophic, even if it is present in larvae before settlement. The two valves are joined together by the ligament along the hinge line and by the massive adductor muscle ventroposteriorly (Figure 3). Its contraction controls valve opening. These mechanisms permit the regulation of the water flow. Oysters are monomyarian molluscs. Adductor muscle consists in two adjacent distinct parts. The fist, sometimes called vitreous muscle, is characterised by a translucent off-white aspect and represents two-thirds of

the total bulk of the muscle. The remainder is crescent-shaped with a pure white opaque aspect. The two parts of the adductor muscle contract at different speeds: the rapid closing of the valves is accomplished by the contraction of the translucent part while the elasticity and tonus of the white part counteract the pulling force of the ligament for long time (Marceau, 1904a, b). Consequently, the adductor muscle is mostly developed in individuals living in the intertidal zone. Finally, short repeated shell movements are observed during spawning or to help the pseudofaeces disposal.

A heart, arteries, veins, and open sinuses constitute the circulatory system that is partially closed. The sinuses, or lacunae, are spaces of varying size, within the connective tissues, without a wall. They are interposed between arteries and veins with a function similar of the capillaries in vertebrates (Galtsoff, 1964). The heart is situated in the pericardium (Figure 3), a thin-walled chamber located between the visceral mass and the adductor muscle. The three-chambered heart consists in a ventricle and two dark-coloured auricles that are covered with a tall columnar epithelium that constitutes a part of the excretory system (Franc, 1960).

The haemolymph is colourless and unlike other mollusc classes, it lacks haemocyanin (Galtsoff, 1964) but contains two main groups of circulating cells termed haemocytes. Haemocytes are not confined to the vessels: they are able to amble throughout the tissues or move to a target in response to a molecular signal.

Exchange of gases takes place essentially in the gills (Figures 3), but the mantle also contributes, even if in a lesser extent, in the respiration. Bivalves maintain a constant water flow through their gills for respiration and feeding. The gills in filter-feeding molluscs are more complex, due to their multiple functions and consist in four folds of tissue suspended from the visceral mass, two for each side.

The digestive system (Figure 4) consists of the mouth, short esophagus, stomach, crystalline style sac, digestive gland, midgut, rectum, and anus. The crystalline style is a peculiar feature of bivalves and some gastropods with a mechanical and enzymatic role in the digestion of food. The stomach is surrounded by the dark-brown digestive gland, made of an important number of tubules emptying in larger ducts leading finally to the stomach. Undigested food is discharged as feces thanks to the ciliary action of epithelium, as peristaltic movements are absent.

The nervous system of oyster is simple. It consists of a primitive system of visceral and cerebral ganglia in which ganglia pairs are connected together through commissures and with

the other pairs through connectives. Several nerves originate from the ganglia and extend to different parts of the body.

To date, the organs of oysters currently known to have sensory function are limited to the short tentacles that fringe the mantle and the pallial organ inside the cloaca. Tentacles contain photo- and chemoreceptors able to detect slight chemical and physical changes in their environment.

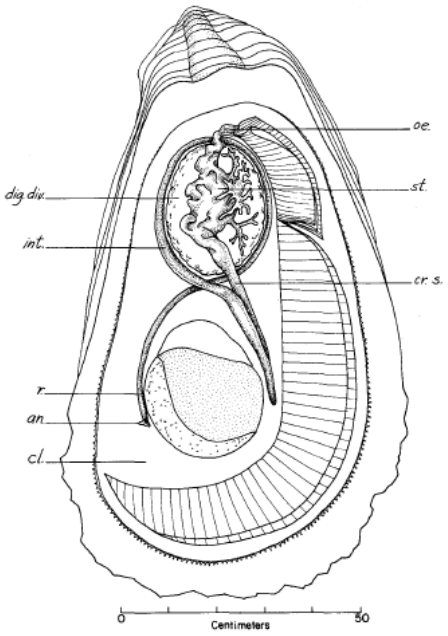


Fig. 4 Diagram of the digestive system of *Crassostrea* (Galtsoff, 1964)
an: anus; cl: cloaca; cr.s: crystalline style sac;dig.div: digestive gland; int: intestine; oe: esophagus; st: stomach

Excretion is carried out by the nephridia, pericardial glands, and also the mantle epithelium, that contributes to this function.

The gonad is located within a connective layer, between the digestive gland and the outer epithelium. In sexually mature oysters, many branching tubules can be observed and converge into a gonoduct. Because Pacific oysters are sequential hermaphrodite, male and female gonads cannot be observed simoultaneously in the same individual.

1.1.4. Reproduction cycle and development

Reproduction is one of the most important physiological processes in the life cycle of bivalve species. *C. gigas* is an alternative sequential hermaphrodite. After settlement, for several weeks, the immature gonad of juveniles remains nonfunctional and ambisexual. It

contains both male and female primary germ cells which will transform into mature spermatozoa or eggs during the following spring-summer. Usually, a predominance of males is observed in the 1-year old population, due to the more rapid multiplication of male germ cells that suppresses the oocyte development. However, the environmental conditions, such as warm waters, are able to influence the number of individuals that develop directly into females (Coe, 1936). During their lifetime, oysters invert sex several times. In wild populations, male are more numerous, with a 5:3 sex-ratio (Le Dantec, 1968).

C. gigas exhibits a seasonal reproductive cycle. In Europe, after spawning, a period of quiescence is observed, in autumn. The restart of gametogenesis is observed in winter, soon or later, depending on the geographic location (Chávez-Villalba *et al*., 2003). In late-winter and spring, oysters tend to accumulate glycogen in their tissues that will be metabolised in lipids during the vitellogenesis. The gonadal development, determined by histological analysis, can be divided into five stages, on the basis of descriptions of Heude-Berthelin *et al.* (2001) (Figure 5). During the stage 0 (quiescent stage), no trace of sexuality is present and only an undifferentiated germinal epithelium is observed; the stage I (early growth stage) is characterised by small follicles and numerous spermatogonia or oogonia; in stage II (late growth stage) the follicles developed actively and a majority of primary gametes with few free spermatozoa and oocytes also are present; stage III is the maturation stage during which gametes are densely packed follicles, filling completely the follicles. At this stage, oocytes are pear-shaped and appear compressed. The vitellogenesis is completed and a distinct nucleus and nucleolus is observed. Spermatozoa are oriented with tails toward the follicle lumen. The stage IV embraces the spawning and the reabsorbing stages and phagocytosis is present.
In Europe, gamete release occurs between July and August, depending on environmental factors (Enríquez-Díaz, 2009) mainly due to the geographic location. Spawning is induced by both sudden fluctuations of physic environmental parameters, temperature in particular, and chemical signals released wit gonadal fluids of other individuals, allowing the synchronisation among individuals. Egg fertilisation occurs in the water medium.

Larvae show planctonic behaviour for a temperature-depending duration. During its planctonic life, the young oysters get through different stages reported in figure 6.

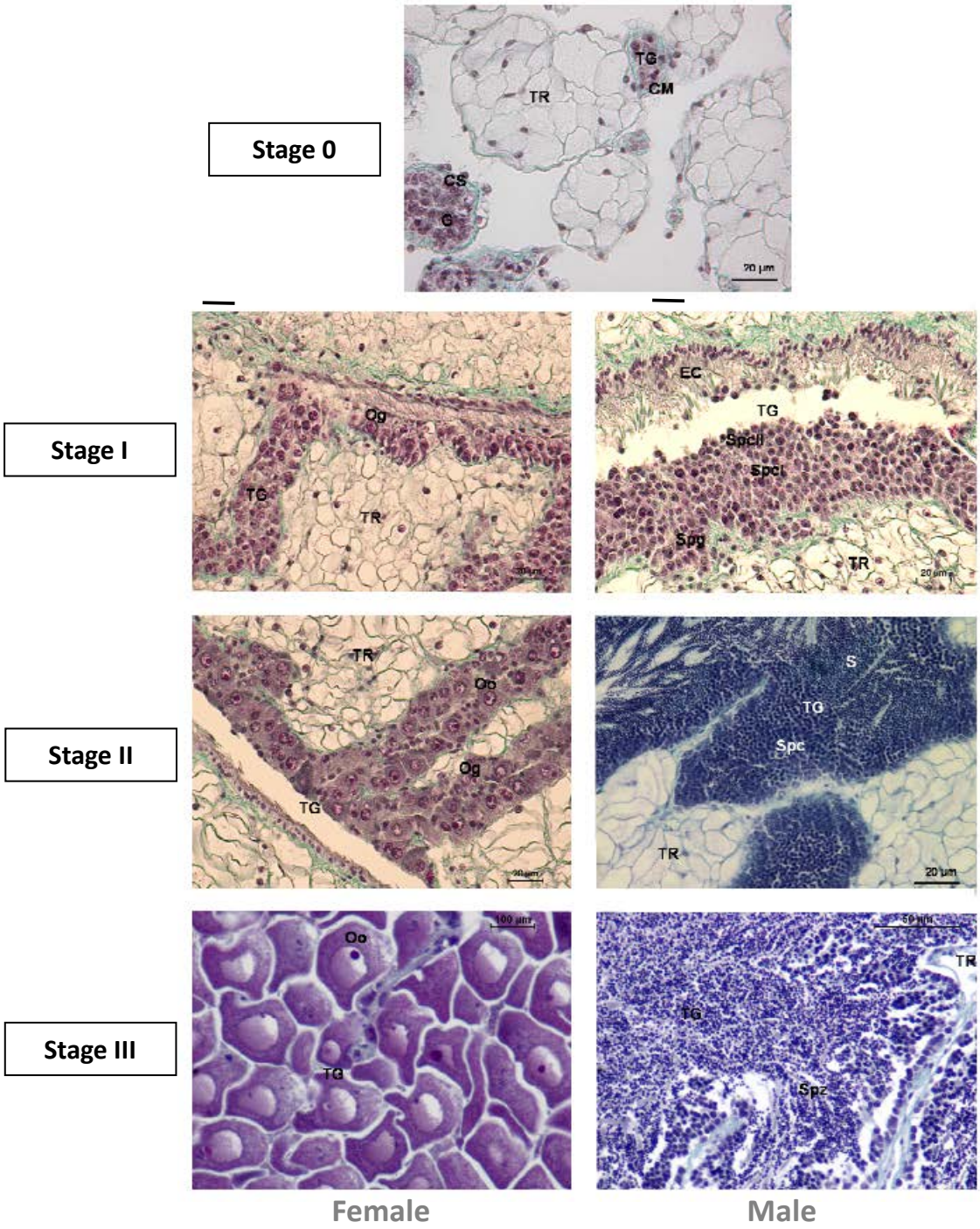
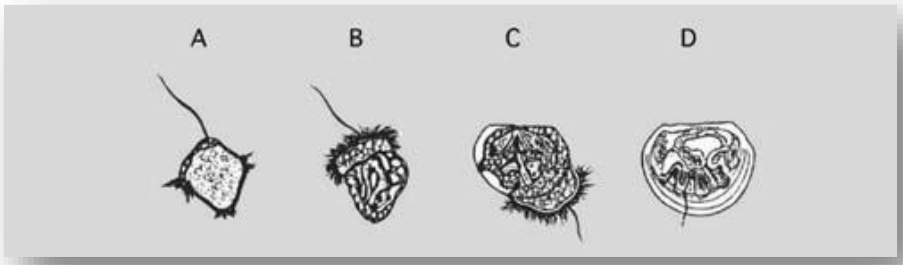


Fig. 5 Photomicrographs of *C. gigas* female and male gonads, stained using Pregnant-Gabe's trichrome.
EC: ciliated epithelium; G: undifferentiated gonia; Oo: oocyte; Og: ovogonia; S: spermatid; Spc: spermatocyte; Spg: spermatogonia; Spz: spermatozoa; TG: gonadic tubule; TR: storage tissue.
(adapted from Jouaux, 2010)



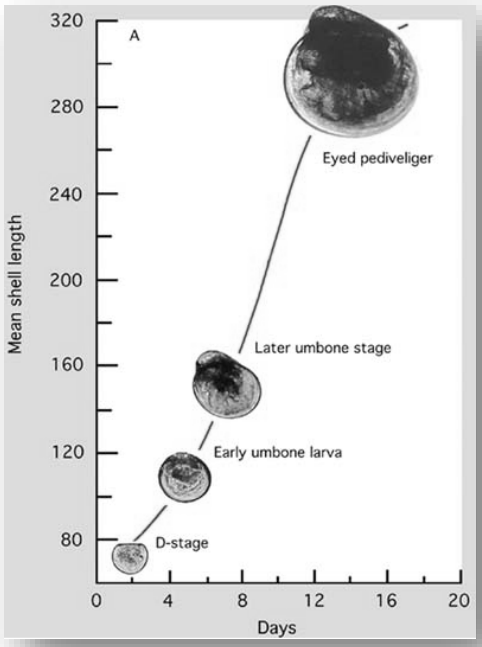


Fig. 6 Developmental stages of *C. gigas* from the early trochophore (**A)** to the fully shelled D-larva stage (**D**). The ciliated swimming feeding organ (velum) can be seen in **B** and early shell valve formation in **C**
http://www.fao.org/docrep/007/y5720e/y5720e0a.htm

At the end of the planctonic stage, a foot is present and helps the pediveliger larva (Figure 6) to crawl searching a suitable substrate to attach to. After settlement, the foot regresses until it disappears completely. At this stage oysters are termed spat.

Fig. 7 Pediveliger larva of *C. gigas* (S. Trancart)

1.1.5. Immune defences

In order to survive in a competitive environment, organisms must be able to protect themselves from pathogens. In these conditions, all metazoan organisms have developed complex immune defence systems. The first line of defence is represented by physicochemical barriers and mucus in particular, that covers all the epithelial layers. The microbiota composition may also contribute to the health status. The adaptive immunity, acquired in vertebrates during evolution (Hoffmann *et al.*, 1999; Hirano *et al.*, 2011), is not present in molluscs, whose set of ancient defence strategies is collectively known as innate immunity, characterised by its universality and rapid-acting. These strategies require firstly an afferent (or sensing) component, able to perceive infection, and secondly an efferent pathway aimed to eradicate infection, and involve both cellular and humoral components. Infection is perceived thanks to the detection of pathogen-associated molecular patterns (PAMPs) (Beutler, 2004). The phagocytosis in invertebrates, evidenced firstly in starfish by Metchnikoff (1884), is the main cell-mediated immune defence. This function is exercised by haemocytes (Lorteau *et al.*, 1995) after their mobilisation and recruitment to the site of infection. Cellular response is coupled with humoral responses: cytokines, enzymes and other immune effectors.

In particular, the knowledge of antiviral defence mechanisms mainly derives from studies conducted on human and vertebrates where innate defences and adaptive immune system collaborate together. This is not the case of molluscs. However, they compensate the lack of adaptive response with a complex innate antiviral defence system that is greatly expanded if compared to vertebrates (Zhang *et al.*, 2013; Venier *et al*., 2011).

Haemocytes

Haemocytes are present in the circulatory torrent, in vessels and sinuses, but also infiltrated throughout the tissues. They also migrate by diapedesis to the surface of epithelial layers. These cells are immunocompetent but they are also involved in different physiological processes, such as gonad resorption, excretion, digestion and transport of nutrients and shell repair (Cheng, 1981). The term haemocyte refers to three main distinct populations of circulating cells: blast-like cells, hyalinocytes, and granulocytes (Hine, 1999; Bachère *et al*., 2004), as shown in Figure 8 thanks to flow cytometry analysis using the Side Scatter (SSC) and Forward Scatter (FSC) parameters. Granulocytes are distinguished from other haemocytes by the possession of cytoplasmic granules and a low nucleus:cytoplasm ratio.

These granules may have different tinctorial properties allowing the subdivision of cells in acidophilic, basophilic, and neutrophilic (Auffret, 1989; Tiscar and Mosca, 2004). Blast-like cells and hyalinocytes are usually classified as agranular haemocytes. The first ones have a spherical or ovoid central nucleus surrounded by a thin rim of cytoplasm while hyalinocytes have a reniform irregular eccentric nucleus and an abundant cytoplasm. To date, the ontogeny of the cell line and the functionality of the different cell types have not been clearly characterised yet. However, even if the main phagocytic activity is assumed to be carried out by granulocytes, hyalinocytes are also able to phagocyte as observed with some protozoan parasites (Chagot *et al*., 1992; Mourton *et al*., 1992). During immune defence, haemocytes perform key role acting in different ways. After the perception of infections or damages due to chemicals, by the afferent component, haemocytic infiltration is observed around the lesion site. In severe infections, haemocytosis is evident in vessels and sinuses (Feng, 1988). Phagocytosis against small particulate such as viruses, bacteria or parasites, described by Cheng (1981), is carried out by haemocytes following different steps: chemotaxis, recognition/binding, engulfing and degradation. A huge respiratory burst is induced after phagocytosis, generating the release of a variety of intermediate ROS (Lambert et al., 2003) with antimicrobial action (Babior, 1984; De Lorgeril et al., 2011). When the dimension of the exogenous element exceeds the phagocytosis capacity, encapsulation has been observed in different molluscs with parasitic diseases (Smolowitz *et al*., 1998; Marino *et al*., 2005). Moreover, the bivalve haemolymph is incapable of clotting, intended as plasma gelation after vessel injury, but haemocytes display a spontaneous property of aggregation, acting as vertebrate platelets, and resulting in cell clots.

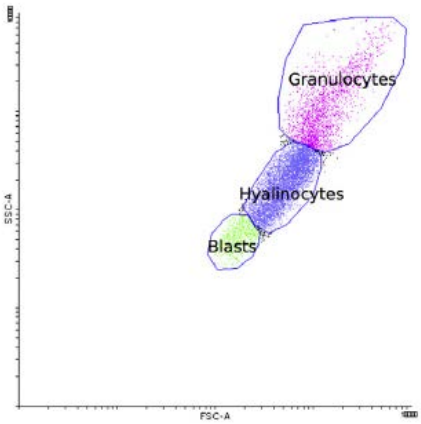


Fig. 8 Cytometry analysis of SSC and FSC of haemolymph whole cells (Bachère *et al.*, 2015)

Toll-like receptors

As in vertebrates, viral PAMPs are recognised by pattern recognition receptors (PPRs) and this association activates complex signaling pathways that lead to the secretion of pro-inflammatory cytokines and antiviral factors. Many components of the innate immunity present in molluscs are conserved in vertebrates, even if PPRs are much more diversified in bivalves, in absence of adaptive immunity. The oyster genome counts a conspicuous number of genes related to immunity aspects such as apoptosis, cytokine activity and inflammatory response (Zhang *et al*., 2012). Through the use of different approaches, the transcriptome response of *C. gigas* to OsHV-1 infections has been investigated (Renault *et al*., 2011; Jouaux *et al*., 2013; Rosani *et al*., 2014; Segarra *et al*., 2014b; He *et al*., 2015). TRLs are transmembrane cell receptors, mainly present on plasma and endosomal membranes, characterised by external leucine-rich repeat domains (LRRs), binding with PAMPs, and by an internal Toll/Interleucine-1 receptor (TIR). The association PAMP/TRL activates TIR that interacts with immune adaptor proteins, such as myeloid differentiation primary response protein 88 (MyD88), leading to the activation of NF-kB. In turn, NF-kB induces the transcription of immune effectors. In mammals, various TRLs recognise different herpesvirus features such as dUTPase and viral DNA/RNAs (Cai and Zheng, 2012; Paludan *et al*., 2011). 83 TRLs have been reported in the oyster genome, many more than in mammals such as human and mice in which only 10 and 12 TRLs are encoded respectively (Leulier and Lemaitre, 2008). Four of these TRLs resulted up-regulated during OsHV-1 infection and may be considered specific for antiherpesviral response (He *at al*., 2015). The four TRLs differ from each other in domain structure and temporal expression: CgTLR-v 1, a plasma membrane TRL, is up-regulated with an expression peak 24 hours post inoculation (hpi), CgTRL-v2 is characterised by the intracytoplasmatic location of LRR and a maximum expression 12 hpi, CgTRL-v3 and CgTRL-v4 are small TRL-like proteins, expressed mainly 6 hpi, lacking LRR and transmembrane domains. Thus, both have cytoplasmic location but their exact function is unknown. Renault *et al.* (2011) and Segarra *et al*. (2014b) observed that MyD88-like adaptors, associated with TRL activation, were also up-regulated. The oyster genome has eight MyD88-like adaptors (Zhang *et al*., 2012), two of which were highly up-regulated. However these two MyD88-like adaptors contain no death domain, suggesting that they may not be able to transduce the signal downstream. This is confirmed by the observation of only a slight up-regulation of down-stream elements (He *et al*., 2015).

Apoptosis

Apoptosis is a physiological process of programmed cell death largely present in nature (Tittel and Steller, 2000), which allows the normal cell turn-over and plays a homeostatic role. However, it is also an important immune response to infective or toxic agents (Elmore, 2007) and it has been evidenced to be particularly developed in *C. gigas*, with 48 genes coding for apoptosis inhibitors, suggesting a high regulation level (Zhang *et al.*, 2012). Two apoptotic pathways are present in molluscs (Terahara and Takahashi, 2008; Sokolava, 2009), including *C. gigas*, and show a high complexity level (Zhang *et al.*, 2011): the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Estévez-Calvar *et al.*, 2013). Capsases are essential components in both. In brief, an initiator capsase, in response to appropriate stimulus, activates an effector capsase, which cleaves several cellular subtrates to induce cell death. Capsase-2, and two effector capsases have been described in the genus *Crassostrea* (Zhan *et al.*, 2014), in particular capsase-3 has been reported in *C. gigas* exposed to cadmium and noradrenaline (Sokolova *et al.*, 2004). In vertebrates, a death transmembrane receptor, member of the TNF receptor gene (TNFRs) superfamily, activates the capsase cascade after recognition of extracellular ligands (Bridgham *et al*., 2003), such as TNF-α. The *C. gigas* genome has expanded TNF and TNFR genes (Guo *et al*., 2015), up-regulated during OsHV-1 infection (He *et al*., 2015). TNF-α homologous has been also described in other members of the family *Ostreidae* such as *O. edulis* (Martín-Gómez *et al*., 2014), together with members of the TNFR family in *Chlamys* (Li *et al.*, 2009) and *Mytilus* (Philipp *et al*., 2012). Nevertheless, different other ligands have been described in *Crassostrea* (Yang and Wu, 2010; Zhang *et al.*, 2011, 2015), TRAIL (Apo2-ligands) and the Fas-ligand in particular. It has also been shown in *C. gigas* that the death receptor pathway in haemocytes is also induced following cell-adhesion mediated by integrin-like molecules (Terahara *et al*., 2005). Extrinsic apoptosis is in turn regulated *via* the NF-κB pathway and all the key components of the anti-apoptotic signaling cascade (NF-κB-IκB-IAP) have been found in molluscs (Montagnani *et al*., 2004). The intrinsic apoptosis pathway is activated after exposure to environmental stress such as the presence of toxicants or radiation exposure, UV for instance, inducing genotoxic damages (Steinert, 1996), presence of cytokines, ROS production, viral parasitism, etc., representing a set of non-receptor-mediated stimuli. In vertebrates, these stimuli act on the permeabilisation of the mitochondria outer membrane that consequently induces the release of pro-apoptosis factors in the cytosol, mainly cytochrome c. The regulation of the apoptotic event in mitochondria is carried out through Bcl-2 (Cory and Adams, 2002) and BI-1 (Robinson *et al*., 2011) genes. Members of the Bcl-2 family have been described in *C. gigas*

(Zhang *et al.*, 2011, 2015) and BI-1 in mussels (Estévez-Calvar *et al*., 2013). In molluscs, as in humans, the implication of the tumor suppressor p53 gene has also been demonstrated, for instance during UV-exposure (Estévez-Calvar *et al*., 2013). Inhibitor of apoptosis proteins (IAP), that are characterised by the presence of Baculoviral IAP Repeat (BIR) domains, are able to induce a negative control on apoptosis interacting with capsases (Deveraux and Reed, 1999) and have been largely evidenced in oyster with the presence of 48 IAP genes (Zhang *et al.*, 2011, 2015). Some pathogens are also able to inhibit apoptosis, preventing cell death. It is the case in several viruses, in order to accomplish their complete replication cycle or during the latent phase such as in herpes simplex virus (HSV) (Perng *et al*., 2000).

RNA interference

The RNA interference is a defence mechanism essentially directed to viruses and is present in plants and animals. The evidence of microRNAs and up-regulation of ribonuclease genes with OsHV-1 infection in *C. gigas* (Xu *et al*., 2014; He *et al*., 2015) suggest that RNA interference is probably a defence pathway in molluscs (Segarra *et al*., 2014a), as observed during viral infections in other invertebrates like shrimps (Robalino *et al.*, 2005). Seven genes encoding retinoic acid-inducible gene (RIG) receptors (RLRs) have been evidenced in *C. gigas* genome and several were up-regulated during OsHV-1 infection (He *et al*., 2015). RLRs are a group of PRRs able to recognise viral dsRNA and trigger an inflammatory response (Cagliani *et al*., 2014). An oyster homolog of the double-stranded RNA-specific adenosine deaminase (ADAR) was also up-regulated (95 times the control) during OsHV-1 infection (He *et al*., 2015), with a peak at 24 hpi. ADAR is an enzyme involved in mRNA editing, altering host and viral gene expression and function. For instance an up-regulation of this gene is observed during hepatitis D virus infection in humans (Jayan and Casey, 2002) leading to the inhibition of viral replication.

Interferons

To date, no interferon (IFN) homologs, a class of cytokines inducing vertebrate cell into an antiviral state (Randall and Goodbourn, 2008), has been found in invertebrates such as oysters (He *et al*., 2015), suggesting that the IFN pathway is a vertebrate innovation (Loker *et al*., 2004). However, transcriptomic analyses on *C. gigas* infected by OsHV-1 revealed the presence of key components of an IFN pathway (Renault *et al*., 2011; Rosani *et al.*, 2014; Segarra *et al*., 2014b; He *et al*., 2015), such as IFN-stimulated gene (ISGs) and interferon regulatory factors (IFRs). The upregulation of ISGs has been evidenced miming viral

infection through poly(I:C) injection (Green *et al*., 2014). In fact, the induction of these genes leads to the successive inhibition of OsHV-1 replication in the same individuals (Green and Montagnani, 2013; Green *et al*., 2014). In particular, oyster viperin results to be one of the earliest upregulated genes during viral infections in *C. gigas* (Green *et al*., 2014; Rosani *et al*., 2014) and it seems to be able to inhibit other virus replication such as dengue virus (DENV-2) replication (Green *et al*., 2015).

Oxidative burst

Oxidative burst consists in the generation of reactive oxygen species (ROS) to degrade pathogens or macromolecules, such as what happens in microglial cell during herpesvirus simplex infection (Schachtele *et al*., 2010). Several oxidase genes are up-regulated during OsHV-1 infection (He *et al*., 2015), with a peak at 24 hpi: three cytochrome P450 oxidase genes, two multicopper oxidase genes, as observed by Renault et al. (2011) also, and a spermine oxidase gene. Unlike what occurs in vertebrates, the main generation of ROS in *C. gigas* probably not lies in NADPH oxidases. In fact, Nox2 homologues were not found in oyster genome, and Nox3 and Nox5 were not up-regulated by the presence of OsHV-1 (He *et al*., 2015). In concert with up-regulation of pro-oxidative genes, several genes encoding antioxidant enzymes are down-regulated during OsHV-1 infection (Corporeau *et al*., 2014; He *et al*., 2015). This concerns a glutathione peroxidase (GPX) gene, eight glutathione S-transferase (GSTs) genes, three catalase genes, two peroxiredoxin-like genes, and five extracellular superoxide dismutases (SODs).

An excessive ROS production can also induce irreversible host cell damages leading to cell death and tissue injury.

Other defences

The role of proteinase regulation is unclear. The action of host protease inhibitors can limit the potential damage caused by pathogens. In fact, proteases are common pathogen virulence factors, promoting the tissue invasion by microorganisms, such as *Perkinsus marinus* (La Peyre *et al*., 1996) or vibrios (Hasegawa *et al*., 2009). However, during OsHV-1 µVar infection, the five metalloproteinase genes are up-regulated while the five genes encoding tissue inhibitors of metalloproteinases are down-regulated (He *et al*., 2015). This increased production of proteinases may induce the destruction of viral or damaged host proteins.

The presence of active antimicrobial peptides and proteins (AMPs) has been evidenced in oysters, with common features with other Phylum AMPs (see review Bachère *et al.*, 2015). In *C. gigas*, in particular, defensins (Gueguen *et al*., 2006; Gonzalez *et al*., 2007a), proline-rich peptides (Schmitt *et al*., 2012), molluscidin (Seo *et al*., 2013b), bactericidal/permeability increasing proteins (Gonzalez *et al*., 2007b), macrophage expressed gene 1-like protein (He *et al*., 2011) and other potentially antimicrobial molecules, such as lysozyme (Takahashi *et al*., 1986), ubiquitin (Seo *et al*., 2013a), and histones (Kawasaki and Iwamuro, 2008) have been characterised. To date, it is unknown if invertebrate AMPs can play a role in antiviral defence.

1.1.6. Triploidy

The majority of *Metazoa* is diploid. It means that somatic cells possess a karyotype with a paired set of chromosomes (2n), for instance n=10 in *C. gigas*. However, polyploidy, a generic term referring to specimens with more than two sets of chromosomes, occurs in nature and it is involved in evolution mechanisms (Hegarty and Hiscock, 2007). Thus, triploidy has been especially observed in plants (review Ramsey and Schemske, 1998) and it is estimated that between 30% and 50% of angiosperm species are polyploid (Grant, 1981). Even if it may also occur in animals (Mable, 2004), and mainly in insects, amphibians (Stöck *et al.*, 2002), and fish, this character is infrequent (Orr, 1990). Burton and Husband (2000) observed that this alteration induces deep consequences on the individual physiology, which usually cause a lower fitness in natural environment. The main changes concern gene expression (see reviews by Adams and Wendel, 2005; Chen and Ni, 2006; Birchler *et al.*, 2007) and disturbance in cell division. Moreover, unpaired polyploidy, such as triploidy (3n), is even less common in nature. Nevertheless, it has been successfully induced in several fish species (see review by Pifarrer *et al*., 2009). In fact, triploid specimens are usually sterile (Allen and Stanley, 1978) and this feature is very favourable for production. In this way, no energy is devoted to gonadal development, in favour of somatic growth. The induction of triploidy was also carried out on different shellfish species, such as the bivalves *C. virginica*, *C. gigas*, *Chlamys varia*, *Mytilus* spp., *Pecten maxima*, and *T. philippinarum* and also gastropods such as *Haliotis discus hannai*, since the early 1980s (see review by Beaumont and Fairbrother, 1991; Pifarrer *et al*., 2009). From an economical point of view, the use of sterile triploid oysters has three main positive implications: as a consequence of what exposed above, triploids grow faster (Nell, 2002), they enable all year-round consumption, and they are assumed to evade the physiological stress induced by reproduction. In fact, during late

spring and summer, the significant development of the gonad and the depletion of glycogen stores affect the organoleptic properties of cupped oysters, making them unmarketable for most consumers. Remembering that *C. gigas* is considered in Europe as a non-native invasive species but also an important economic activity, an additional positive aspect in favour of the use of sterile triploids is the containment of its diffusion in nature. For all these reasons, a survey conducted in 2008 showed that triploid seed produced in hatcheries represented 78.7% of the spat produced in hatcheries in France (Cultures Marines n°23, février 2009).

The induction of triploidy in the genus *Crassostrea* was first performed in *C. virginica* by Stanley *et al.* (1981), through the use of cytochalasin-B. The same method was then carried out for the production of triploid *C. gigas*. At the spawning stage, mollusc oocytes are arrested at the prophase or metaphase of Meiosis I (Colas and Dubé, 1998). The egg fecundation induces the resumption of the meiosis just after the spermatozoa entry and the extrusion of the two polar bodies (Figure 9).

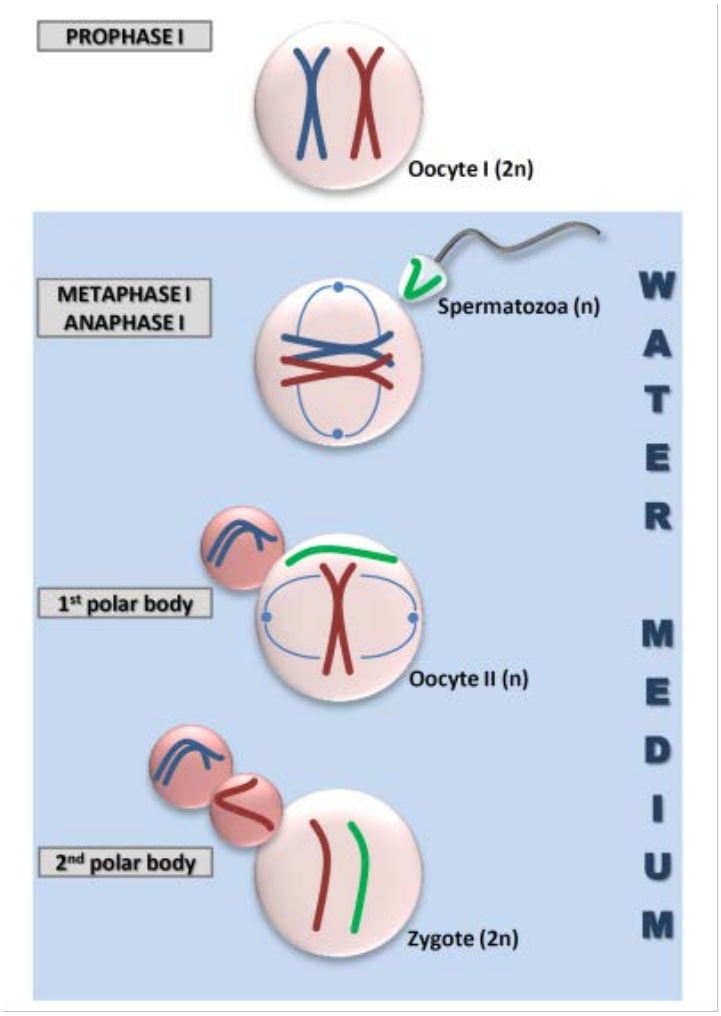


Fig. 9 Normal fertilisation events in diploid x diploid crossing.

Physical or chemical shocks, during Meiosis I or Meiosis II, are able to prevent the extrusion of a polar body, producing triploids (Figure 10). Usually, the treatment is applied during the release of the second polar body because it ensures better efficiency and larva survival (Guo *et al*., 1992). However, the high toxicity of cytochalasin-B to operators and animals (Pitts, 1994), the insufficient rates of survival and triploidisation in treated larvae led to the development of other methods based on the obtainment and use of tetraploid fertile males for reproduction. Diploid sperm, from tetraploid males, is used to fertilise normal haploid oocytes, generating a triploid line (Guo *et al*., 1996; McCombie *et al*., 2005) (Figure 11). To date, different methods are used to obtain tetraploid individuals, and two patents for the production of tetraploid males have been recorded.

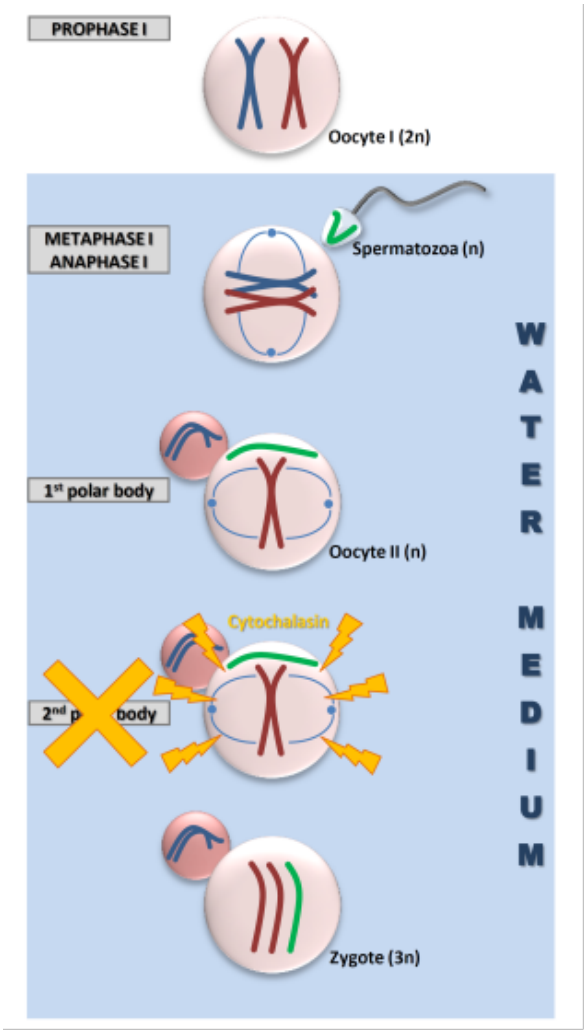


Fig. 10 Chemical induction of triploidy in oysters.

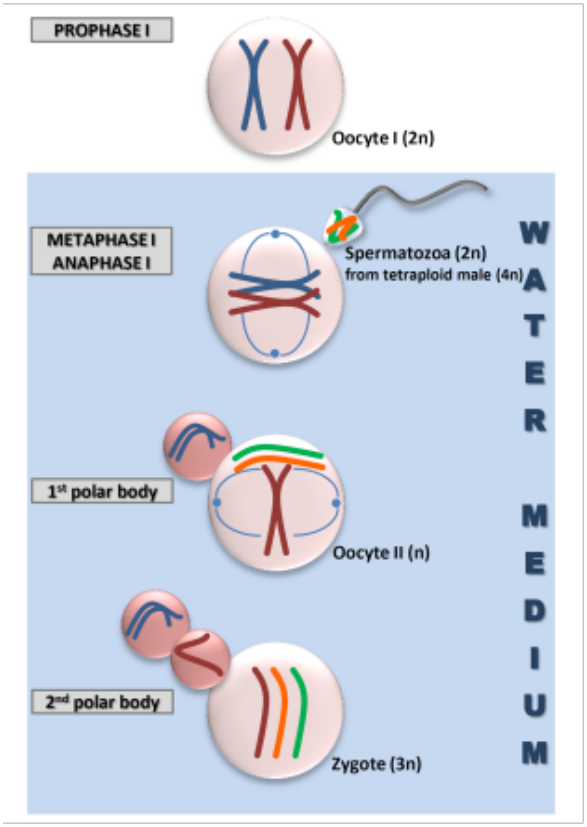


Fig. 11 Obtainment of triploid individuals using sperm from tetraploid males.

In shellfish, triploidy does not necessarily produce complete sterility, but rather a decrease of gonadal development. Allen and Downing (1986) reported a probable spawning in triploids produced by treating newly fertilises eggs with cytochalasin B. In particular, environmental conditions seem to influence the degree of gonadal development as reported in France during the 2003 heatwave (Normand *et al*., 2008). Gametogenesis has been also observed in triploids issued from tetraploid x diploid crossing and a new classification, was suggested to describe gametogenesis in triploid oysters (Jouaux *et al*., 2010): the specimens displaying germinal cell proliferation are called α individuals and those producing only few gametes are termed β individuals. However, to date, the effect of the different methods to obtain triploid specimens on gametogenesis capabilities has not been evaluated yet.

One of the main problems that interferes with the triploid gametogenesis is the chromosomic segregation (Figure 12). In fact, during the meiosis I, homolog chromosomes are divided randomly between the daughter cells: two in one and only one in the other (Gong *et al*., 2004). The result is the generation of a majority of aneuploid gametes, with an average of 15 (n=10 + 5) chromosomes (Normand, 2005).

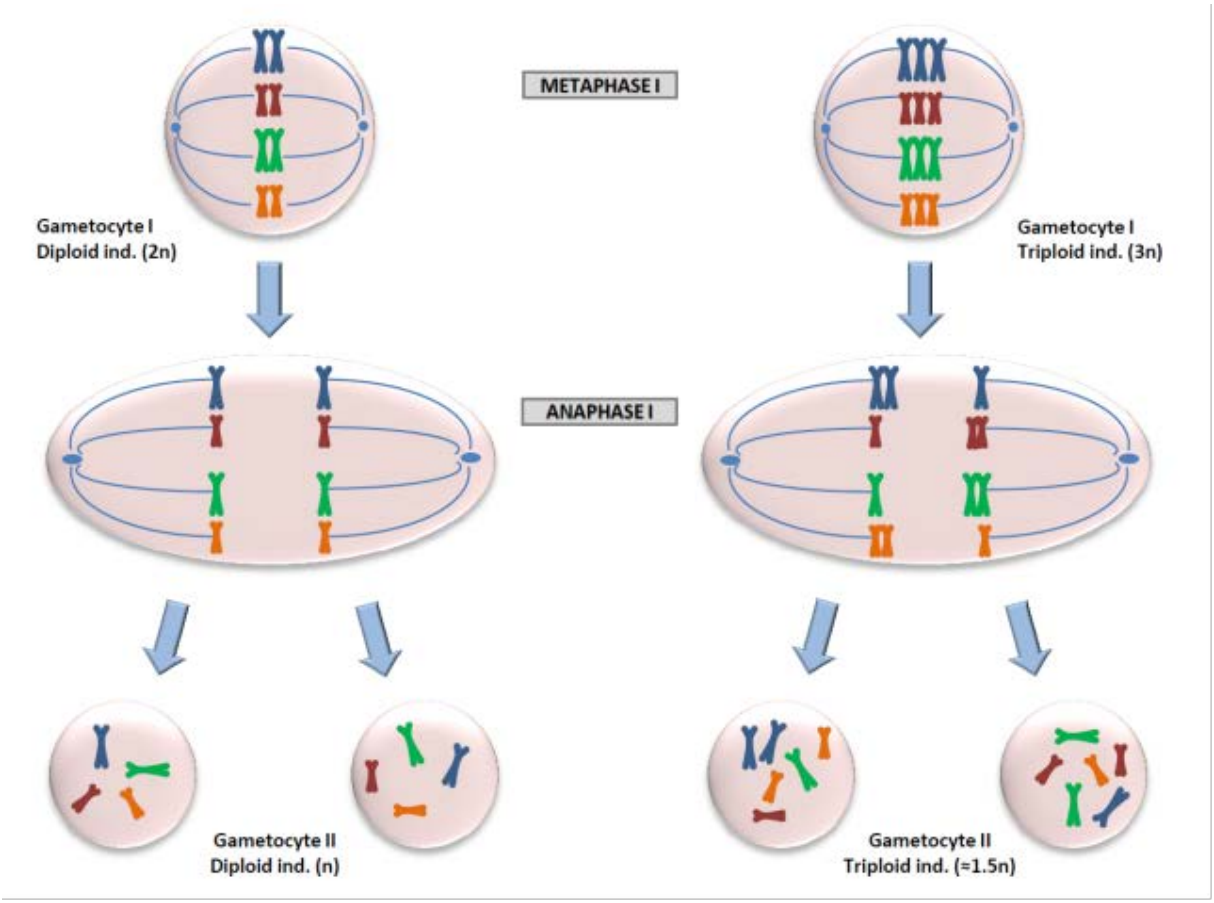


Fig. 12 Comparison of chromosome segregation during Meiosis I and resulting gametocytes II in diploid and triploid individuals.

Fertilisation crossing triploid and diploid individuals is successful but the resulting zygote is usually aneuploid, compromising survival (Guo and Allen, 1994).

1.1.7. Production cycle

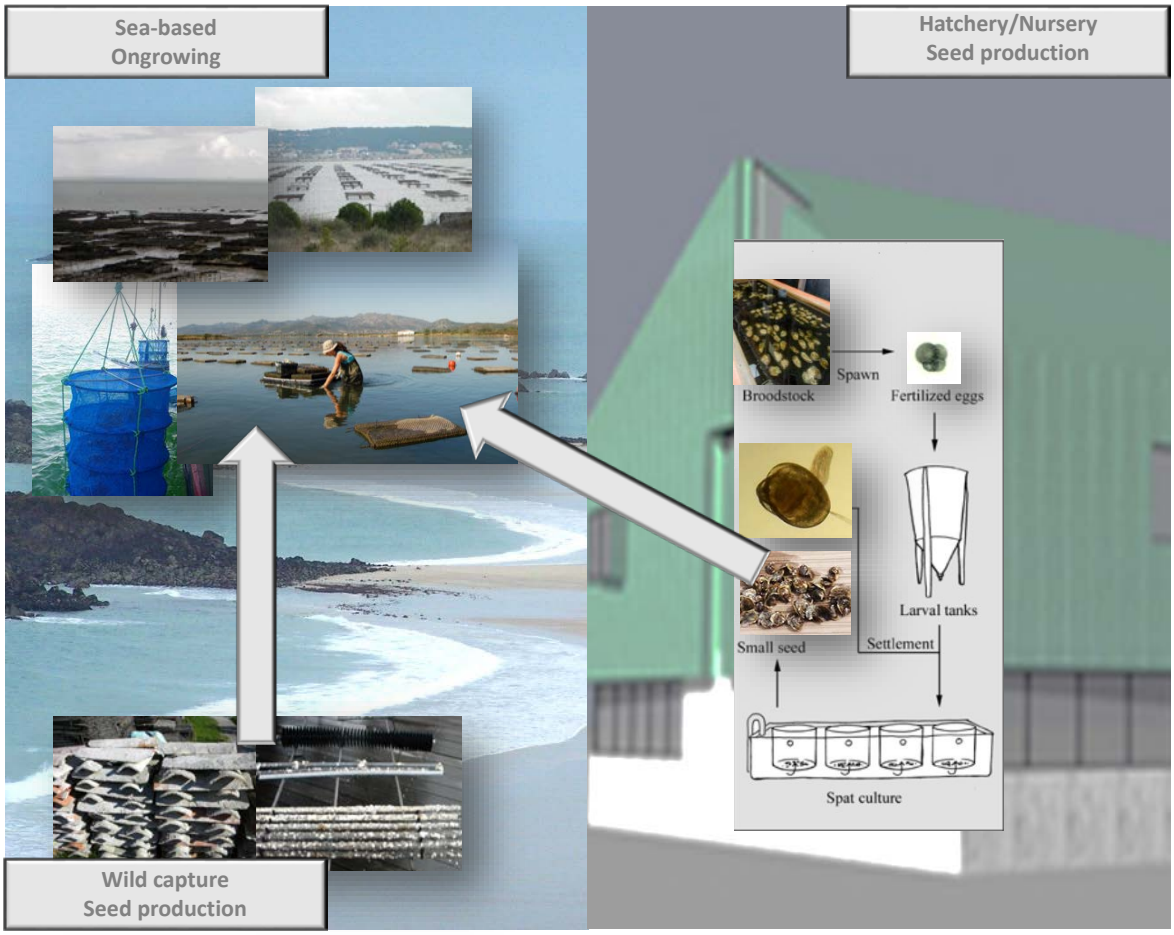


Fig. 13 Common producing cycle of *C. gigas* in Europe, from seed to commercial product.

The rearing cycle of the Pacific oyster extends for an average period of three years in France (Figure 13). The cycle is shorter in warm waters and lasts about two years in the Mediterranean area. For the seed supply, two main sources are available, issued from different production methods. When the supply of natural spat is abundant and reliable, such as in the Arcachon Bay in France where natural collection is a traditional activity, oyster growers set out spat collectors in the wild, using a wide variety of settlement materials. However, in order to be less dependent of natural spawning seasonality and fluctuations in seed availability, the production of seed is also carried out in artificial conditions in hatcheries. These commercial units represent also the unique suppliers of triploid individuals. After a period in closely

controlled conditions within the hatchery facility, from spawning to settlement and until a minimum length of the individuals of about 2-5 mm, the growth of small seed is conducted in a nursery, often land-based and directly supplied by marine waters, or sea-based using upweller systems (FLUPSY) until a common length of about 15 mm. Then, from now on, grow-out is almost entirely sea-based and the rearing techniques were developed to adapt to the environmental constraints, while feeding is naturally fulfilled through the filtration of sea water particulate. A subsequent phase of ripening is sometimes conducted, only in some producing areas, to improve the organoleptic characterisation of the final product.

1.1.8. Mass mortality events affecting C. gigas

The control of diseases in natural environments, especially marine conditions, is very arduous and oyster production is periodically threatened. Even if young stages of farmed animals are usually more susceptible than adults, both classes of oysters are concerned by mortality events.

During summer 1991, and in the subsequent years, *C. gigas* larvae have been affected by mortality occurrence in French hatcheries (Nicolas *et al.*, 1992; Renault *et al.*, 1994 a,b) and simultaneously, analogous events have been reported in New Zealand (Hine *et al.*, 1992). These episodes were characterised by high mortality rates (90-100%) and associated with infection by a herpes-like virus. The implication of *Vibrio* infections in these phenomena, *V. splendidus* strains in particular, was not excluded (Lacoste *et al*., 2001; Le Roux *et al*., 2002). Since 1993, mortalities have extended to farming sites, in spat, with mortality rates from 80 to 90%, always in summer and associated with the observation of herpes-like particles in mantle and gills of moribund individuals (Renault *et al.*, 1994b). These observations were confirmed thanks to the development of a PCR that permits to better diagnose the presence of the virus (Renault *et al*., 2000). Experimental transmission of the disease demonstrated the pathogenicity of the viral agent, with 100% mortality occurring within 6 days after infection (Le Deuff *et al*., 1994; Renault *et al.*, 1995). The virus was described (Le Deuff and Renault, 1999; Davison *et al.*, 2005), classified as a member of the order *Herpesvirales* (Davison *et al*., 2009) and the species termed *Ostreid herpesvirus* (Minson *et al*., 2000). Ostreid herpesvirus type 1 (OsHV-1) was associated with similar events in California (USA) (Friedman *et al.*, 2005), Mexico (Vasquez-Yeomans *et al.*, 2004), Ireland and Jersey (Peeler *et al.*, 2012), and Australia (Jenkins *et al.*, 2013) involving both larval and juvenile stages.

Unfortunately, a dramatic intensification of the mortality phenomena has been observed since April-May 2008 (Cochennec-Laureau *et al.*, 2009; Renault *et al*., 2009). The mortality rates ranged between 60% and 100%, with a very acute trend, and a rapid diffusion to all the producing regions in France and subsequently to other European countries and in the rest of the world. During these events a new variant of OsHV-1 called µVar was detected (Segarra *et al*., 2010). In the following years, the µVar and several other closely-related genotypes, subsumed under the term "microvariants" (OIE, 2014), were described in *C. gigas* around the world, such as in Australia (Jenkins *et al*., 2013; Paul-Pont *et al*., 2014), China (Renault *et al*., 2012), France (Martenot *et al*., 2011, 2012; Renault *et al*., 2012), Ireland (Lynch *et al*., 2012; Peeler *et al*., 2012; Morrissey *et al*., 2015), Italy (Dundon *et al*., 2011; Burioli *et al*., 2016), Japan (Shimahara *et al*., 2012), Mexico (Grijalva-Chon *et al*., 2013), Netherlands (Gittenberger *et al*., 2016), New Zealand (Keeling *et al*., 2014), Norway and Sweden (Mortensen *et al*., 2016), South Korea (Hwang *et al*., 2013) and Spain (Roque *et al*., 2012). An OsHV-1 microvariant was also associated with mortality events in adult individuals of *C. angulata* in Portugal (Batista *et al*., 2015). A complementary role of bacteria in the development of the disease is highly suspected as demonstrated by Petton *et al*. (2015b). The mortality in Specific-Pathogen-Free (SPF) juveniles of *C. gigas* (Petton *et al*., 2013) treated with chloramphenicol resulted in a 2- to 4- fold decrease in mortality if compared to untreated batches, even in presence of very high viral load.

Adult oysters are also affected by mass mortality events. During summer months, episodes have been described since the 1950s in France, Japan, and USA (Imai *et al*., 1965; Maurer *et al*., 1986; Beattie e*t al*., 1988; Goulletquer *et al*., 1998; Costil *et al*., 2005). The syndrome was termed "summer mortality", because of the probable influence of different factors, such as the physiological stress associated with gonadal maturation mainly (Samain *et al*., 2007), the presence of pathogenic vibrios such as *V. aestuarianus* and members of the Splendidus clade (Garnier *et al*., 2007; 2008), and environmental conditions (Soletchnik *et al*., 2007). Finally, since 2012, a recrudescence of mortalities in adults is observed in France and in Italy (Repamo, 2013; Burioli *et al*., 2015). The seasonality of the disease has changed with an extension and translation of the critical period later in the year (REPAMO, 2013).

Figure 14 represents the seasonality of mortality events in the different age classes through the graphical representation of the number of sampling carried out by the REPAMO (REseau de PAthologie des MOllusques) network during mortality episodes in 2012. It is evident that mortality in spat (≤12 month aged) and juveniles (13-24 month aged) is

concentrated between May and July, while in adults they are mainly observed from August to November.

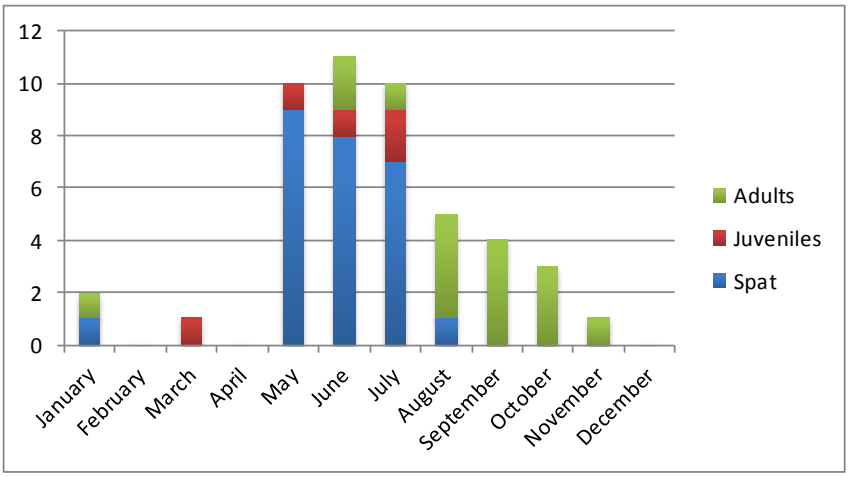


Fig. 14 Comparative number of *C. gigas* sampling in response to mortality reports during 2012 (adapted from Repamo, 2013).

As exposed above, the aetiology of these events is uncertain and complicate and different exogenous factors must be considered (Figure15), together with intrinsic factors from the host. Many factors (climate warming, pollution, harvesting conditions, introduced pathogens) can contribute to disease outbreaks in marine life. In particular, environmental conditions, such as temperature and salinity may represent cofactors in the insurgence of diseases, especially if we consider the present period as affected by climate changes. Stressful conditions, next to tolerance limits, may deplete the host resistance and also promote the pathogen microorganism replication. Oysters are usually farmed in coastal areas and, for instance, an influx of freshwater from rivers during rainy periods may induce a decrease in salinity and the affluence of toxic substances such as pesticides from agriculture, industrial products, and heavy metals. The farming conditions are also a decisive risk factor: the constant increase of the stocking density in the rearing sites is an additional element of stress for animals.

Fig. 15 Exogenous factors influencing oyster health status.

Even if they do not seem to be involved in the recent outbreaks, other pathogens are known to induce harmful diseases in Pacific oysters. One of the most important, because of its high pathogenicity, is *Mikrocytos mackini*, responsible for the disease called Denman Island Disease, a protistan of uncertain taxonomy, fortunately still not present in Europe (listed as exotic disease in the annex IV, part II, DIRECTIVE 2006/88/EC). The pathogenicity of *Nocardia crassostrea*, an Actinomycete bacterium, seems to be weaker, even it has been reported during mortality events in Japan and USA (Numachi *et al*., 1965; Friedman *et al*., 1991). It has also been signalised in Europe, in the Netherlands (Engelsma *et al*., 2008).

Finally, because of their omnipresence in marine environment, the association between vibrios and diseases is complicate, with high risk of thoughtless interpretations. Nevertheless, the already mentioned *V. aestuarianus* and *V. splendidus* are not the unique species showing pathogenic capability. After their isolation during mortality events, *V. tubiashii* and *V. crassostreae* strains have been able to induce mortalities in experimental infections (Takahashi *et al*. 2000; Elston *et al*., 2008; Travers *et al*., 2014; Lemire *et al*., 2015). *Vibrio harveyi* is also suspected to have consequences on the *C. gigas* survival (Saulnier *et al*., 2009).

1.2. Ostreid herpesvirus type 1 (OsHV-1)

1.2.1. Viral infections in bivalve molluscs

Viral infections have been described in different mollusc species and often associated with mortality events especially in farmed stocks. In fact, the rearing conditions probably facilitate the insurgence of diseases and, above all, make possible their observation. However, in absence of mollusc cell lines and molecular tools for the diagnosis of most of them, the study of these viruses is mainly based on electron microscopy investigations (see review from Renault and Novoa, 2004).

To date, the most relevant viruses known to affect bivalves belong to the family *Malacoherpesviridae* or are related to the families *Iridoviridae*, *Picornaviridae*, and *Retrovoridae.* Retroviruses have been suspected to be involved in the etiology of the so-called "disseminated neoplasia" (Elston *et al*., 1992) observed in various bivalve species, such as *Mya arenaria* (House *et al.*, 1998) and *Mytilus* spp. (see review from Barber, 2004), even if this association has not been confirmed yet. During distinct mortality episodes, the presence of *Picornaviridae*-like viruses has been detected, for instance in the Japanese pearl oyster *Pinctada fucata martensii* (Miyazaki *et al.*, 1999), in the carpet-shell clam *T. decussatus* (Novoa and Figueras, 2000), and in the cockle *Cerastoderma edule* (Carballal *et al.*, 2003). In the blue mussel *M. edulis* and in the pearl oyster *Pinctada margaritifera* the presence of picorna-like particles were associated with chronic inflammatory conditions evidenced by the presence of granulocytomas (Rasmussen, 1986; Comps *et al.*, 1999). Nevertheless, to the best of our knowledge, the members of the *Iridoviridae* and *Malacoerpesviridae* represent the greatest threats for the genus *Crassostrea.* Irido-like viruses (Figure 16) are retained to be the causative agent of the epizootic mortalities that conduced to the phasing-out of the Portuguese oyster *C. angulata* farming in France and to the probable eradication of the species along the French coast.

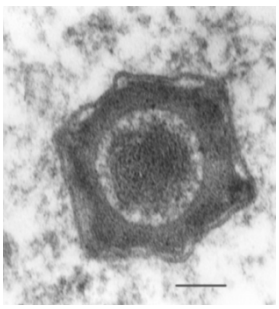


Fig. 16 Irido-like virion from *Crassostrea angulata* in TEM. Scale bar = 100 nm (from Renault and Novoa, 2004).

Two symptomatologies have been observed in this species. During the 1966-1968 outbreaks, occurred in France, necrotic lesions mainly located in gills and labial palps have been detected, so that the agent was termed gill necrosis virus (GNV) (Alderman and Gras, 1969), even if mantle and adductor muscle were also concerned by necrosis (Comps, 1970b). In the individuals affected by the subsequent outbreaks, reported again from 1970, no lesions were observed in gills but the presence of anomalous haemocytes, showing intracytoplasmatic inclusion bodies gave rise to the suspect of the presence of a second irido-like virus that was named haemocyte infection virus (HIV). In adult Pacific oyster *C. gigas*, the diseases induced by the GNV and HIV have been retained to evolve in a milder form (Comps and Duthoit, 1979). On the contrary, the oyster velar virus disease (OVVD), also associated with an irido-like virus, has been reported during occasional mortality events in pediveliger larvae of this species between 1976 and 1984, in a hatchery from the Washington state (USA) (Elston, 1979; Elston and Wilkinson, 1985). The larvae exhibited exfoliation of the ciliated epithelium of the velum that may appear as marginal blebs. These lesions compromise the swimming activities.

1.2.2. Herpesviruses in molluscs

The first herpes-like virus infection in an invertebrate species was described in 1970 (by EM) in the Eastern oyster *C. virginica*, thanks to the observation of intranuclear inclusions with viral particles (Farley *et al.*, 1972). It is only twenty years after that similar viral particles have been detected firstly in *C. gigas*, in France (Nicolas *et al.*, 1992) and New-Zealand (Hine *et al.*, 1992), and subsequently in other bivalve species around the world, often during mortality events. Infections by herpesviruses concern mainly young stages (larvae and spat) of cupped oysters *C. gigas* and *C. angulata* (Arzul *et al.*, 2001c), European and Antipodean flat oysters *O. edulis* (Comps and Cochennec, 1993) and *Ostrea chilensis* (Philippi, 1844) (Hine *et al.*, 1998), Manila clam *R. philippinarum* (Renault *et al.*, 2001), scallop *P. maxima* (Renault and Lipart, 1998; Arzul *et al.*, 2001a;b). In few cases, in French hatcheries and nurseries, the larvae and spat of different species present in the facility were affected by the disease at the same time (Renault and Lipart, 1998; Renault *et al.*, 2000).

In china, herpesviruses have also been associated with mortality events in adult specimens of *Chlamys farreri* infected with AVNV (Wang *et al.*, 2002), which is supposed to be a variant of OsHV-1 or a very closely related species (Ren *et al.*, 2013). During these events mortality reached 90%. Disease in adult individuals has been also observed associated

with OsHV-1 in *Scapharca broughtonii* broodstocks (Xia *et al*., 2015) and in *C. angulata* (Batista *et al*., 2015). Replication of herpes-like viruses has also been reported in adult Chilean flat oysters *Ostrea angasi* (Sowerby, 1871) that experienced low level of mortality in Australia (Hine and Thorne, 1997), during co-infection with the parasite *Bonamia* sp.

Moreover, infection of marine gastropods, abalones in particular, by herpesviruses such as "abalone herpesvirus" AbHV-1 have been commonly described. In various species such as *Haliotis diversicolor supertaxa* (Chang *et al*., 2005), *H. laevigata*, and *H. rubra rubra* (Tan *et al*., 2007), herpesvirus infection induces a fatal disease termed "Abalone Viral Ganglioneuritis" (AVG).

1.2.3. Taxonomic aspects of Herpesvirales

To date, the order *Herpesvirales* clusters more than 100 species (ICTV; www.ictvonline.org) that affect numerous vertebrate classes, from mammals to fish, but also invertebrates as recently stated in the description of OsHV-1 and AbHV-1. Consequently, the classification of herpesviruses has been updated with the definition of three families, as reported by Davison *et al*. (2009). The family *Herpesviridae* incorporates viruses of reptiles, birds and mammals, the family *Alloherpesviridae* retains viruses from fish and frogs, and the new family *Malacoherpesviridae* contains the mollusc viruses classified into two genera: *Ostreavirus* and *Aurivirus,* with a single known species each: OsHV-1 and AbHV-1 respectively. The revised classification is shown in Figure 17. The large family *Herpesviridae* has been split in three subfamilies: *Alphaherpesvirinae* (for instance, herpes simplex virus type 1 HSV-1, varicella zoster virus VZV, equine herpesvirus type 1 EHV-1, bovine herpesvirus type 1 BoHV-2, suid herpesvirus SuHV-1), *Betaherpesvirinae* (such as human cytomegalovirus HCMV) and *Gammaherpesvirinae* (such as Epstein-Barr virus EBV and equine hespesvirus type 2 EHV-2).

Herpesvirus species infecting vertebrates are usually associated with one precise host species but in some special conditions, such as farming or more generally in environments promoting the promiscuity between individuals of different species, the transmission to another related species is possible. In this form of infection, the pathogen is often much more virulent for the non-natural host. Probably, this phenomenon is imputable to host and pathogen co-evolution. For instance, infection with *Cercopithecine herpesvirus* 1, also termed B-virus, in the natural host the macaque monkey is usually asymptomatic, but when humans are infected they can develop a severe encephalomyelitis. This zoonosis resulted fatal

in >70% of the known cases (Huff and Barry, 2003). Similarly, Aujeszky's disease, even termed pseudorabies and caused by SuHV-1, is acutely fatal in dogs (Monroe, 1989) but induces less than 5% of mortality in adult pigs, the natural host (Wittmann, 1986). On the contrary, infections with OsHV-1 have been described in numerous bivalve species, as detailed previously.

The proteins encoded by OsHV-1 show a low homology with those of vertebrate herpesviruses except for the ATPase subunit of the terminase, whose sequences have been compared by phylogenetic analysis, evidencing well-separate branches for each *Herpesvirales* family (Savin *et al.*, 2010). The terminase is believed specific of herpesviruses and bacteriophages such as T4, being an enzyme involved in packaging viral DNA into preformed capsids (Davison, 2002).

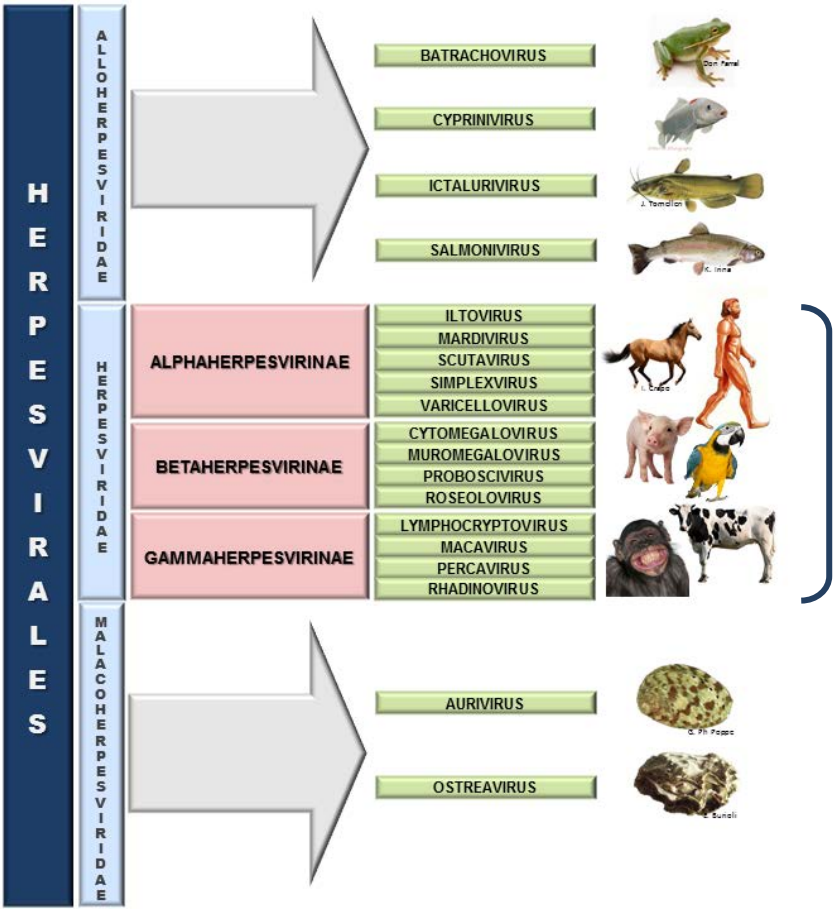


Fig. 17 ICTV's classification of herpesviruses.

1.2.4. Biological characteristics

Similarities exist between the *Herpesvirales*:

- Encoding of specific enzymes, such as protein-kinase, thymidine-kinase, dUTPase, ribonucleotid reductase, DNA polymerase, helicase, primase, and terminase.
- Capsid assembly in nuclear location.
- Viral latency ability: during the latent phase no clinical symptoms are noticeable in the host, latent genome may integrate into cellular DNA or be present in an episomic form in the nucleus, and only few genes, mainly responsible for the maintenance of the latency phase, are expressed (Thiry *et al*., 1986; Garcia-Blanco and Cullen, 1991). The virus reactivation is induced by stress-related stimuli. Usually, only few restricted cell types are the site of latency (see review Grinde, 2013), especially nervous or lymphoid cells. To date, the latency ability of OsHV-1 in its host has not been clarified, even if it is highly suspected in asymptomatic adult oysters (Arzul *et al*., 2002). The finding of the abalone neural ganglia as the site of latency in AbHV-1, another member of the family *Malacoherpesviridae*, strengthens this hypothesis.

Few characteristics vary between the different herpesvirus species. For instance, some of them are able to infect a large number of cell types, while others target a specific cell type. The different studies, conducted through electron microscopy, ISH, or IHC in larvae and spat of different bivalve species during mortalities, reported the presence of herpes-like particles or positive signals in “interstitial cells”, “fibroblast-like cells”, and haemocytes in the connective tissue of different organs (Nicolas *et al*., 1992; Renault *et al*., 1994a; Renault *et al*., 2001; Arzul *et al*., 2002) and also in epithelial cells (Martenot *et al*., 2016). Heart, mantle and gills may be potential sites for primary replication (Segarra *et al*., 2016). Other differences involve the replication cycle length: short in *Alphaherpesvirinae* with a productive cycle of less than 24h (Matthews, 1982), whilst is at least 48 hours in *Betaherpesvirinae*. OsHV-1 is believed to have a short replication cycle, insofar as during experimental infections conducted in larvae by bathing in virus suspensions, all individuals were moribund within 48 hours and viral particles were observed in cell nuclei (Le Deuff *et al*., 1994). Moreover, some OsHV-1 transcripts such as those encoding DNA polymerase and IAPs appeared to be present 2 hours post infection (Segarra *et al*., 2014a).

1.2.5. General structure of herpesviruses

The general structure of OsHV-1 is complex and similar in overall appearance to those of other herpesviruses (Figure 18). The multilayered architecture consists in a capsid, enclosing a DNA core in a highly condensed form, surrounded by a thick proteinaceous matrix called tegument, and then by a lipidic envelop with surface spike, derived from host cell-membrane and including virus-encoded proteins. Capsid is a rigid structure that protects the viral DNA from damages and releases the nucleic acid molecule into the host cell nucleus. The capsid of herpesviruses is a well-defined icosahedron (20-faceted) (T=16), composed of four distinct structural proteins. The major capsid protein (MJC) is the basic monomer that constitutes the 162 capsomers: 150 hexons and 12 pentons at the vertices. All hexons have the same protein composition and result from six MJC monomers. Pentons have two different compositions: eleven consist of five MJC monomers and one, called portal vertex, is a ring-shaped structure composed of a non-structural protein complex allowing the entry of viral DNA within the capsid (Newcomb *et al*., 2001). A heterotrimeric complex, termed triplex, composed of two distinct proteins, one present in two copies, connects the capsomers. The fourth protein, which is the smallest, forms hexamers attached to each hexon (Zhou *et al*., 1995). This continuous ring is not present on the top of pentons. The capsid diameter of OsHV-1, estimated from cryo-electron microscopic images, is about 116 nm (Davison *et al.*, 2005), slightly smaller than other herpesvirus (~125 nm) (Roizman and Pellett, 2001). The tegument of herpesviruses represents a significant part of the virion space and contains approximately 40% of the herpesvirus virion protein mass (Gibson, 1996). It consists of various proteins, asymmetrically distributed in the space between capsid and envelop, that are delivered to cells at the very initial stage of infection and are important for various aspects of the viral cycle, playing a key role both before and after the activation of the viral genome. They are involved in transport of the capsid throughout the cytoplasm to the nuclear pore, immune response, regulation of viral transcription, regulation of apoptosis, secondary envelopment, etc (see review Kelly *et al*., 2009). It is generally agreed that more than 20 proteins constitute the *Alphaherpesvirinae* tegument (Loret *et al*., 2008). The envelope is the external trilaminar structure and it gives round to pleomorphic morphology to herpes virions. It derives from a cell membrane, probably from the Golgi apparatus in the case of OsHV-1 (Hine *et al.*, 1992), modified by the insertion of viral proteins and glycoproteins. Glycoproteins are essential during the infection process, being involved in the attachment and

the penetration of the virus in cells but also during the exit from cell phase (Bentz, 1993). The exact number of membrane glycoproteins in OsHV-1 has not been determined yet. However, in *Alphaherpesvidae*, for instance, 11 glycoproteins are highly conserved and necessary for the accomplishment of the virus cycle (Roizman and Pellett, 2001). In addition to the viral encoded glycoproteins, the envelope also contains numerous host proteins or constituents.

The determination of the exact virion size is complex because it depends on the tegument thickness that varies during the maturation cycle of the virion and ranges from 120 to 300 nm. The treatments, used to process the samples for electron microscopy observation, also influence the final size of the viral particle.

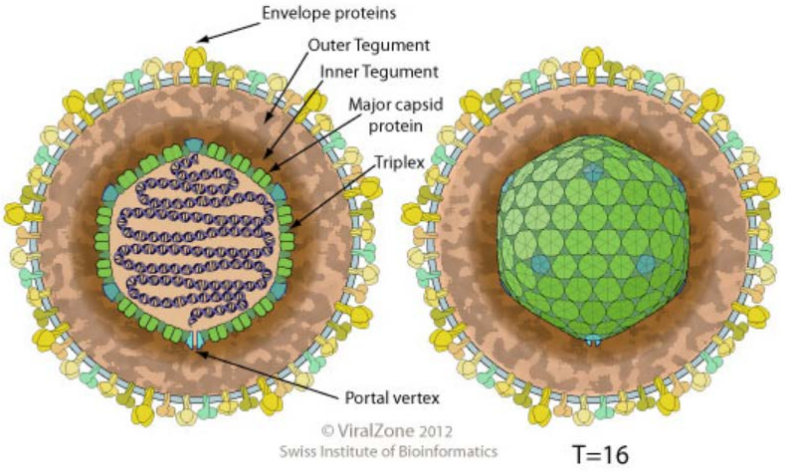


Fig. 18 Structure of a herpesvirus particle.

1.2.6. Genome arrangement of OsHV-1

The genome of herpesviruses consists of a linear double-stranded DNA molecule with a length comprised between 125 and 290 kbp (Pellett and Roizman, 2006) and G+C contents between 32% and 75% (Roizman *et al*., 1992). The complete genome of OsHV-1 has been sequenced by Davison *et al*. (2005) (GenBank Ay509253). The total genome length is 207,439 bp and the nucleotide composition is 38.7% G+C (Davison *et al*., 2005). A feature of herpesviruses is the presence of repeated sequences that may be inversely oriented. Roizman and Pellett (2001) classified *Herpesviridae*, independently from viral families, in six classes from “A to F” on the basis of their genome organisation (Figure 19). Only one species, the tupaïa herpesvirus (THV), belongs to the class F. The genome reference of OsHV-1 is represented in Figure 20 and is close to class E, even if the *a* region (region X in OsHV-1) is

usually present in single copy in OsHV-1. As determined by Davison *et al*. (2005), it contains two unique regions, U_L (167,863 bp) and U_S (3,370 bp), each flanked by a large inverted repeat TR_L/IR_L (7,584 bp) and TR_S/IR_S (9,774 bp), and an internal third unique X region (1,510 bp). This particular genome organisation leads to four possible isomers of OsHV-1, apparently present in equimolar amounts, as observed in some mammal herpesviruses, such as HSV-1 and HCMV (Hayward *et al*., 1975; Westsrate *et al*., 1980.

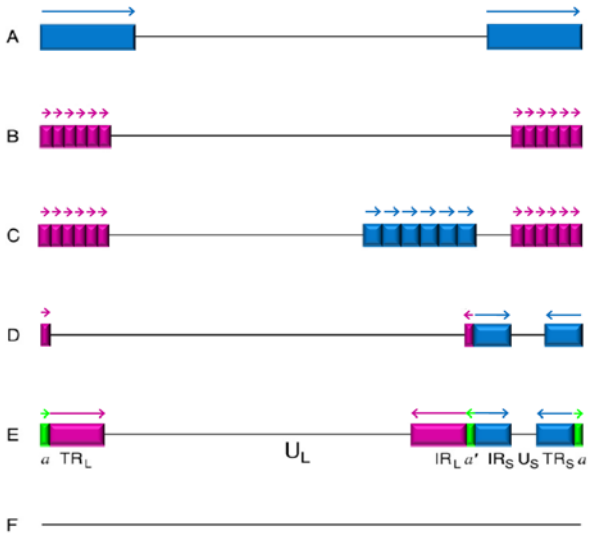


Fig. 19 Genome organisation of *Herpesviridae* within six classes.

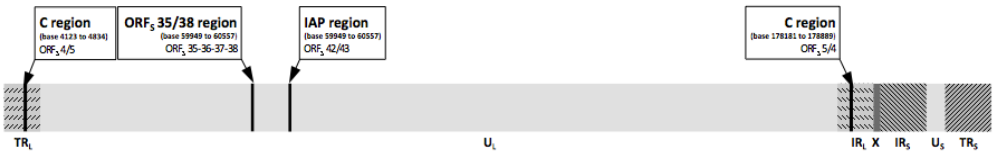


Fig. 20 Organisation of the OsHV-1 genome.
U_L, U_S, and X: unique regions; TRL/IRL and TRS/IRS: inverted repeats.

A total of 124 open reading frames ORFs, corresponding to unique protein-coding genes, has been predicted (Davison *et al*., 2009) (Figure 21), 12 of which duplicated because they are located in the inverted repeats. No indications of splicing emerged from the Davison's analysis. As reported in vertebrate herpesviruses, especially in those with larger

genomes (Chee et *al.*, 1990), families of related genes (12) were highlighted in OsHV-1 (Figure 21). A putative function or domain was appointed to 40% of genes. About 14% were supposed to encode membrane proteins, 10% enzymes, 7% proteins with RING finger domains, and 3% apoptosis inhibitors (IAPs). These putative IAPs belong to a family of viral and cellular proteins known as “baculovirus inhibitor of apoptosis repeat proteins” (BIRPs) and two of them contain also a RING finger. BIRPs have been evidenced in large DNA viruses of invertebrates, *Ascoviridae* (Stasiak *et al*., 2000), *Asfaviridae* (Yáñez *et al*., 1995), *Baculoviridae* (Crook *et al.*, 1993), *Iridoviridae* (Jakob *et al*., 2001), and *Poxviridae* (Afonso *et al*., 1999), and they are probably used to subvert host apoptotic defences. Functional information was provided for other genes encoding a putative primase, two subunits of ribonucleotide reductase, a helicase, the catalytic subunit of a polymerase δ, and the ATPase subunit of terminase. Finally, eight genes appear fragmented that is a current observation in many members of the *Poxviruses* (see review Gubser *et al*., 2004) but still undescribed in herpesviruses.

A large palindrome involving about 200 bp is situated in U_L. By analogy with some vertebrate herpesviruses, the palindrome is a candidate for an origin of DNA replication (Weller *et al*., 1985; Inoue *et al*., 1994).

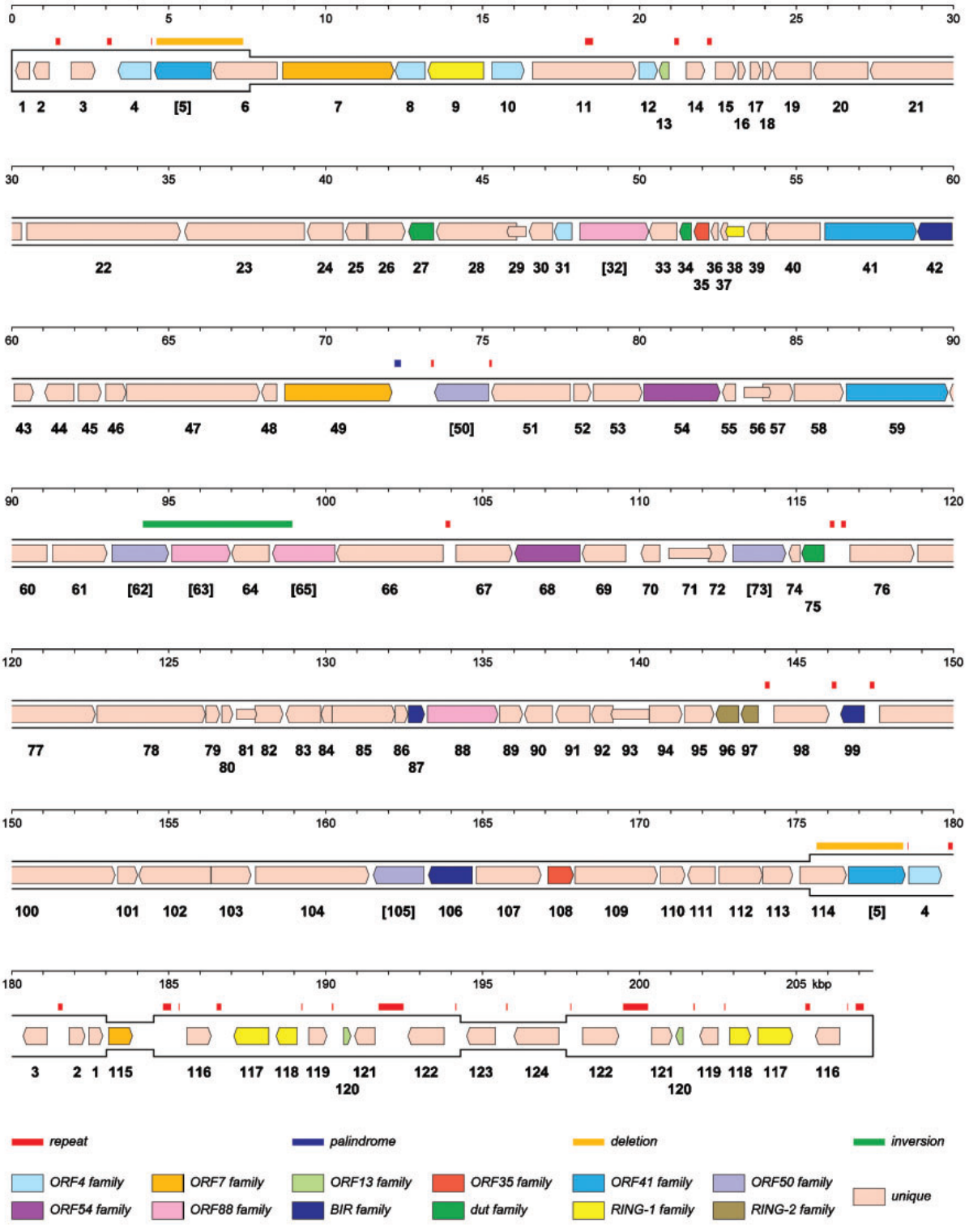


Fig. 21 Layout of ORFs in the OsHV-1 genome. Colours are used to identify the 12 genes families. Scale in kbp. Square brackets apply to fragmented genes depicted as intact. (Davison *et al*., 2005)

1.2.7. Genetic diversity of OsHV-1

The genomic diversity in double-stranded DNA viruses like herpesviruses is quite low due to the proofreading capability of DNA polymerases. Until 2001, only two genotypes were known: the reference type (Davison et al., 2005) and a second genotype termed OsHV-1 Var evidenced in *C. gigas*, *R. philippinarum*, and *P. maximus* larvae during mortality events (Arzul, 2001; Arzul *et al.*, 2001a, Arzul *et al.*, 2001b), characterised by the deletion of 2800 bp in the TR_L region. Several variants have since then been described by sequencing specific areas of the genome. The C region was the most variable area. In 2008, simultaneously with the increase in oyster mass mortality events in France, another variant was isolated and called µVar (Segarra *et al.*, 2010). It is formally characterised by a deletion of 12 consecutive bp in the C region (GenBank HQ842610) in a microsatellite locus (OIE, 2014). Other mutations are present in the µVar genotype: in ORFs 42/43 encoding a IAP, and in most cases a deletion of 605 bp, corresponding to the total loss of both ORFs 36 and 37 and the partial loss of ORF 38 encoding a RING finger protein (Segarra *et al.*, 2010; Renault *et al.*, 2012). Although the µVar appears to be the most common genotype in C. gigas in France since 2008 (REPAMO, 2010), several other new variants have been described, mainly on the basis of the C region analysis, in France (Martenot *et al.*, 2011, 2012; Renault *et al.*, 2012), Ireland (Lynch *et al.*, 2012; Morrissey *et al.*, 2015), New Zealand (MPI, 2010; Renault *et al.*, 2012), Mexico (Grijalva-Chon *et al.*, 2013), China (Renault *et al.*, 2012), South Korea (Hwang *et al.*, 2013), Australia (Jenkins *et al.*, 2013), and Japan (Shimahara *et al.*, 2012). Retrospective studies have demonstrated that the µVar genotype was already present in 2004 in Normandy (France) (Martenot *et al.*, 2012) and in New Zealand in 2005 (OIE, 2014) but its origin remains uncertain. Interestingly, Martenot *et al.* (2013) reported the presence of five large deletions in the genome of µVar specimens isolated between 2009 and 2011 in Normandy and Ireland. Finally, related genotypes infecting other bivalve mollusc species in China (Ren *et al.*, 2013), such as the viral necrosis virus (AVNV) (GenBank GQ153938) in *Chlamys farreri* and the OsHV-1-SB strain (GenBank KP412538) associated with mortality events in *Scapharca broughtonii* broodstocks, have been described and their complete genome sequenced.

1.2.8. Replication cycle of OsHV-1

Since bivalve cell lines are not yet available (Yoshino *et al.*, 2013) and heterologous cell lines are unfit for the propagation of OsHV-1, the information on virus replication cycle derives mainly from observations conducted on electron microscopy. On the basis of these studies the replication cycle, reported in Figure 22, appears to be very close to what observed in other herpesviruses. The host transcription study based on a microarray assay, conducted by Jouaux *et al*. (2013), gave also important contributes to the comprehension of the OsHV-1 replication cycle.

To enter in the host cell, the virus needs first to bind and fuse to the cell membrane by the recognition of specific viral ligands by host membrane receptors. Herpesviruses usually engage multiple receptors during viral entry (Figure 23). Some are considered binding receptors only that may serve to concentrate virus on the cell surface without inducing changes required for membrane fusion. Others are real entry receptors, binding to which triggers the subsequent events required for membrane fusion. The presence of different potential receptors increases the spectrum of infectable cell types. For instance, in *Alphaherpesvirinae*, three classes of entry receptors have been identified: the process of infection begins when the virions bind heparan sulfate moieties on cell surface proteoglycans (Shieh *et al.*, 1992; Shukla *et al.*, 1999; 2001; Spear *et al*., 2000; Tiwari *et al.*, 2006) and then fuse with the herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor family, or with nectin-1 and nectin-2, two members of the immunoglobulin superfamily. In *Gammaherpesvirinae*, viral glycoproteins may interact with multiple lymphocyte B surface determinants such as the complement receptor 2, involved in the attachment phase (Kieff and Rickinson, 2001), and HLA class II molecules (Li *et al*., 1997) or integrins for the fusion step in EBV and HHV-8 (Akula *et al.*, 2002) respectively. To date, the recognition pathway is not known in OsHV-1. However, the implication of cellular glycipan, a cell surface heparan sulfate proteoglycan (Segarra *et al.*, 2014b), and integrins (Jouaux *et al*., 2013) is highly suspected. The entry into the cell seems actin-dependent (Jouaux *et al*., 2013).

The nucleocapsid translocation to the nucleus, through the cytoplasm by the cytoskeleton (Lyman and Enquist, 2009), provides for the microtubular network, such as α and β tubulins, and probably actin fibers participation, also in OsHV-1 (Roberts and Baines, 2011; Jouaux *et al*., 2013). Reached the nuclear pore, the viral genome is released within the nucleus.

When conditions are favorable for the virus, the productive cycle is activated. The nucleolus appears enlarged and nuclear changes including hypertrophy, chromatin margination and pycnosis are observed but Cowdry type A inclusions are not a diagnostic feature during OsHV-1 infections (Arzul, 2001). The exact evolution of the replication cycle in OsHV-1 is unknown but usually the herpesvirus synthesis is orchestrated through the sequential expression of three classes of genes: the immediate early (IE), early (E), and late (La) genes (Honess and Roizman, 1974). IEs encode α proteins, which functions are the regulation of the E genes and are almost only expressed during the first hours of replication cycle in HSV-1. The E genes encode β proteins, mainly responsible for the viral DNA replication and maturation, such as DNA polymerase and helicase. β proteins act also to down-regulate α proteins. Finally, L genes encode γ proteins comprising capsid structural proteins and accessory proteins required for its assembly. The herpesvirus genome replication starts previous DNA circularisation and firstly occurs through a theta replication mechanism followed by the rolling cycle replication process (Ben-Porat and Tokezewsky, 1977; Severini *et al*., 1996). The last, induces the synthesis of a long DNA monomer that is subsequently cleaved and associated with proteins yielding to single mature viral nucleoids. The capsid assembly occurs in the nucleus. Then, the nucleoid is encapsidated passing through the portal vertex and anchors to the inner capsid surface thanks to protein fibers forming the nucleocapsid (Furlong *et al*., 1972). By budding through the inner nuclear membrane to the perinuclear space, the virus acquires the primary envelope, immediately lost crossing the outer nuclear membrane. It seems that budding occurs in nuclear membrane areas spiked by virus-encoded glycoproteins. The second envelopment leads to the acquisition of the definitive virion envelope, adorned with the binding glycoproteins. Presumably, in OsHV-1 infections, this phase occurs when nucleocapsids bud into Golgi cisternae (Hine *et al*., 1992). The tegument components are acquired passing by this various cellular compartments. Enveloped virions are finally released at the cell surface.

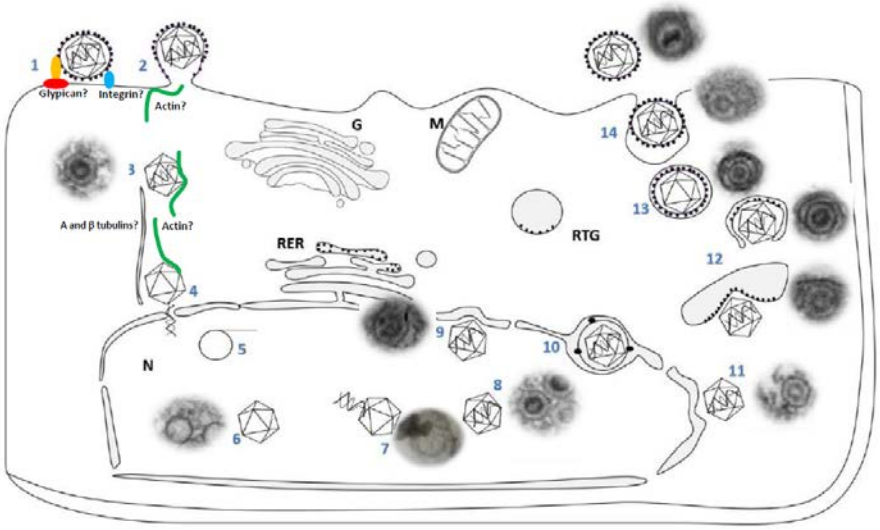


Fig. 22 Hypothetic replication cycle of OsHV-1 (adapted from Segarra, 2014):
1. binding; 2. fusion and entry; 3. tegument activation; 4. transport to nuclear pore; 5. viral DNA replication; 6. capsid assembly; 7. DNA incapsidation; 8. nucleocapsid; 9. fusion with the inner nuclear membrane; 10. primary enveloped particle; 11. de-envelopment and liberation in the cytoplasm; 12. envelopment in a Golgi cisterna; 13. transport to the plasma membrane; 14. exocytosis of mature virion. G, Golgi complex; M, mitochondrion; N, nucleus; RER, rough endoplasmic reticulum; RTG, Golgi vesicles.

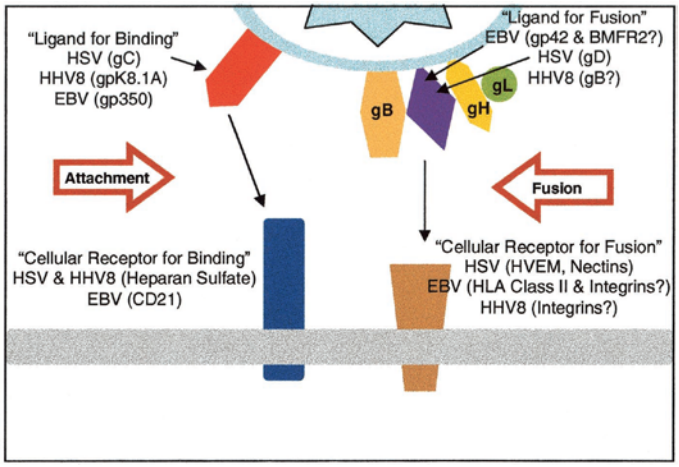


Fig. 23 Participants in herpesvirus (HSV, HHV-8, EBV) entry and virus-induced cell fusion (Spear and Longnecker, 2003).

1.2.9. OsHV-1 transmission and resistance in environment

As the presence of the virus in gonads has been evidenced using ISH and IHC (Arzul *et al*., 2002), vertical transmission of OsHV-1 is suspected (OIE, 2014) but has not been demonstrated yet.

Horizontal transmission through seawater has instead been shown in different studies. The cohabitation of experimentally infected oysters with naïve oysters led to the induction of mortality and high viral loads in naïve oyster while a constant concentration of viral DNA copies/µL ranging between 10 and 10^3 in the tank water was observed trial-long (Schikorski *et al*., 2011a). The same result was obtained with frozen dead OsHV-1 infected oysters conserved at -80°C for six months, showing that the infectivity was conserved (Paul-Pont *et al*., 2015).

Whittington *et al*. (2015) observed that the mortality in spat was reduced to zero by filtering OsHV-1 naturally contaminated seawater with a 5µm filter before its entry in the nursery, but not with a 30µm filter. During these trials, in the control tanks supplied with unfiltered water, mortality reached 60-100%. The same result as filtering was obtained by aging water for 48 hours. These two protocols may act positively removing viruses attached to particles, by sedimentation and filtration. These observations are consistent with particulate-attached virus theory (Paul-Pont *et al*., 2013a; Evans *et al*., 2014). The adsorption of viruses onto the surfaces or suspended particles is a well-known phenomenon, especially in aquatic environments (Bitton, 1975; Sakoda *et al*., 1997; Lyons and Ward, 2005). Thanks to qPCR, Wang *et al*. (2010) showed that AVNV associates with several species of marine microalgae. The positive effect of water aging may also result from the loss of infectivity after 48h. Recently, Martenot *et al*. (2015) demonstrated that OsHV-1 µVar remains infectious in sea water after 54 h at 16°C, and 33 h at 25°C. These results are consistent with those of Hick *et al*. (2016) that evidenced how the virus infectivity is maintained in seawater for 2 days at 20°C and for at least 7 days at 20°C in wet or dry oyster tissue. Moreover, Paul-Pont *et al*. (2015) demonstrated that a purified virus inoculum, consisting in a seawater viral suspension, stocked for 3 months at 4°C, conserves its infectivity. However, these studies used artificial sterile seawater, so these time scales may vary in the field. In another herpesvirus affecting mollusc, AbHV, infectivity conservation in seawater at 15°C was established in few days only (Corbeil *et al.*, 2012) and even less time at 25°C. Studies conducted in chelonid herpesvirus shows that the infectivity is maintained for 5 days in marine water (Curry *et al*., 2000), demonstrating that marine water influences positively the stability of the virus. The white spot

syndrome virus, a large enveloped DNA virus affecting shrimps, maintains its infectivity for up to 12 days in seawater (Satheesh Kumar *et a*l., 2013). Thanks to these observations we can conclude that dead specimens can represent a risk for the spread of the disease.

The study conducted by Hick *et al*. (2016) evidenced that OsHV-1 virions are inactivated by several commercial disinfectants, including Virkon® S (DuPont), quaternary ammonium compounds, sodium hydroxide (20g/L, 10 min), iodine (0.1%, 5 min) and formalin (10% v/v, 30 min); and by physical treatments such as heating to 50°C for 5 min and exposure to a high dose of UV radiations, as also demonstrated by Schikorski *et al*. (2011b). However, the effect of natural doses of UV exposure on OsHV-1 is unknown. Interestingly, both heating at 45°C for 5 min and the treatment with an alkaline detergent (2000 ppm, 10 min) were inefficient (Hick *et al*., 2016). Sodium hypochlorite (50 ppm available chlorine, 15 min) resulted efficient only in relatively clean water.

To date it is not clear how and where OsHV-1 persists during the coldest months, when no mortality events have been reported. Nevertheless, some environmental factors have been correlated with the expression of the clinical disease and with the consequent virion excretion. One of the most important seems to be the water temperature (Renault *et al*., 1995; Petton *et al*., 2013; 2015b; Renault *et al*., 2014): a threshold temperature of 16°C has been established for the insurgence of mortalities, with a range comprised between 16°C and 24°C (Pernet *et al*., 2012; Petton *et al*., 2013), while in Australia and California mortalities due to OsHV-1 have been observed with temperatures reaching 25°C (Burge *et al*., 2007; Jenkins *et al*., 2013; Paul-Pont *et al*., 2013b).

1.2.10. Genetic-based host resistance to OsHV-1

Susceptibility to the viral infection also depends on intrinsic genetic factors of the host as it was highlighted during the studies conducted by Sauvage *et al*. (2009) and Segarra *et al.* (2014b) that compared different oyster families and showed significant different responses to the OsHV-1 infection and different mortality rates. However, Segarra (2014) has also shown that OsHV-1 is released in environment by less susceptible individuals, asymptomatic, but able to excrete virus particles for ~48 hpi.

1.3.Diagnostic methods for OsHV-1

1.3.1. Clinical signs

None observable sign is pathognomonic for OsHV-1infection in diseased animals. In fact, the course of the disease is acute and the clinical signs are not specific of the infection with OsHV-1. Infected hosts may get slower in closing their valves when disturbed but this behavioural change is not specific to infection with OsHV-1 (OIE, 2014).

1.3.2. Histology

Histological examination of infected tissues is not recommended as a diagnostic tool for OsHV-1 (OIE, 2014) in oysters. Nevertheless, some lesions presumably imputable to a herpesvirus infection have been largely described and concerned mainly nuclear changes including hypertrophy, nuclear margination and pycnosis. These anomalies involved in particular fibroblastic-like cells in connective tissue (Nicolas *et al*., 1992; Renault *et al*., 1994a). Cowdry type A inclusions, which are eosinophilic intranuclear inclusions with perinuclear chromatin, are typical of many herpesvirus infections but they have never been reported for *C. gigas* infected by OsHV-1 (OIE, 2014).

Tissues used for histopathological observation should be fixed for 48 hours in 10% formaldehyde fixative such as Davidson's fixative (22% formaldehyde, 33% ethanol 95, 12% glycerol, 33% of 0.2 µm filtered seawater and 10% acetic acid), embedded with paraffin, and stained with haematoxylin and eosin.

1.3.3. Transmission electron microscopy

Transmission electron microscopy is a useful method to confirm the presence of viral particles in infected animals. It gives information on the viral morphology, location, and it also allows observing its replication cycle within the cell. However, electron microscopy is not recommended as a standalone diagnostic tool for the detection of OsHV-1. In fact, it is not specific, being unable to identify the viral strain definitely, poorly sensitive, and quite laborious.

1.3.4. In situ hybridisation (ISH)

Several ISH methods for detecting mRNAs and DNA of OsHV-1 in histological sections have been developed (Arzul *et al*., 2002; Lipart and Renault, 2002; Corbeil *et al*., 2015). Corbeil *et al*. (2015) showed that RNA probes targeting three ORFs were able to detect the virus mRNAs in paraffin sections of experimentally infected oysters 26 h post-injection. These methods are useful to localise the virus, evidencing the target tissues and replication sites, but they are not recommended for surveillance or diagnostic programs. In fact, they do not allow giving a rapid response and they require samples with high viral load because of their low sensitivity. Moreover, these methods are not able to discriminate the different variants of OsHV-1.

1.3.5. Immunohistochemistry (IHC)

Since no adaptive immune system is present in mollusc, serological diagnosis is not feasible. Thus, direct detection methods have been tested. The first study conducted by Le Deuff *et al*. (1995) showed that antibodies specific for channel catfish virus (CCV) cross-react with OsHV-1. However, the response was very slight and poorly usefull. IHC assays based on the use of polyclonal antibodies have been tested (Arzul *et al*., 2002; Martenot *et al*., 2016). In the Martenot *et al.* (2016) assay, antibodies were produced from recombinant proteins corresponding to two putative membrane proteins and one putative apoptosis inhibitor encoded by ORF 25, 72, and 87, respectively. However, some discordant results with respect to other studies that used alternative methods can be signalled with the antibodies against putative membrane proteins, especially regarding the type of tissues and cells tested positive, raising some suspicion of unspecific ligation.

1.3.6. Polymerase Chain Reactions (PCRs)

Since the first description of OsHV-1, a number of different PCR strategies have been developed for the detection of the virus (Table 3). These methods include both conventional (cPCR) and quantitative real-time PCRs (qPCRs), able or not to discriminate between the reference strain and the microvariants. PCR-based methods have a great analytical advantage in terms of sensitivity and specificity if compared to other techniques (Batista *et al*., 2007; Martenot *et al*., 2010).

The characteristics of each PCR-based diagnostic method are reported in Table 3: type of PCR, gene target, primer name, primer sequences, sensitivity, and discrimination capability between µvar and reference strains.

Briefly, cPCRs present the disadvantages if compared to qPCR of lower sensitivity, higher time-consuming, and impossibility to quantify the sample viral load. However, qPCR is not able to discriminate the microvariants from reference strain.

1.3.7. Virus isolation on cell cultures

To date the isolation of OsHV-1 on cell cultures is not viable: the attempts to culture the virus in both vertebrate and invertebrate cell lines and in primary oyster cell cultures have been unsuccessful (OIE, 2014).

PCR method	Gene target	Formard primer	Sequence 5'-3'	Reverse primer	Sequence 5'-3'	Sensitivity	Matrix	µVar discrimination	References
conventional	unknown protein	OsHV3	unpublished	OsHV4	unpublished	not stated	fresh or frozen mollusc tissue paraffin-embedded tissues	no	Renault and Lipart, 1998
conventional	apoptosis inhibitor	B2	CAACAGCTTTGGGAGGTTGGT	B3	GTGGAGGTGGCTGTTGAAAT	not stated	fresh or frozen mollusc tissue	no	Arzul *et al* ., 2002
conventional	unknown protein	C2	CTCTTTACCATGAAGATACCCACC	C6	GTGCACGGCTTACCATTTTT	2 fg of viral DNA per mg of tissue	fresh or frozen mollusc tissue	yes	Renault and Arzul, 2001 Renault *et al* ., 2004
conventional	unknown protein	C13	CCTCGAGGTAGCTTTTGTCAAG	C5	CCGTGACTTCTATGGGTATGTCAG	100 ng l^{-1} in seawater	seawater	yes	Vigneron *et al* ., 2004
conventional	glycoprotein	Gp3	GGTTGTGGGTTTGGAAATGT	Gp4	GGCGTCCAAACTCGATTAAA	100 ng l-1 in seawater	seawater	no	Arzul *et al* ., 2001a Vigneron *et al.* , 2004
conventional	unknown protein	C9	GAGGGAAATTTGCGAGAGAA	C10	ATCACCGGCAGACGTAGG	not stated	paraffin-embedded tissues	no	Barbosa-Solomieu *et al* ., 2004
conventional	apoptosis inhibitor	B4	ACTGGGATCCGACTGACAAC	B3	GTGGAGGTGGCTGTTGAAAT	not stated	paraffin-embedded tissues	no	Barbosa-Solomieu *et al* ., 2004
conventional	unknown protein	C9	GAGGGAAATTTGCGAGAGAA	C10	ATCACCGGCAGACGTAGG	~5000 GU/50µL purified DNA	fresh or frozen mollusc tissue		Webb *et al* ., 2007
conventional	DNA polymerase	OsHVDPFor	ATTGATGATGTGGATAATCTGTG	OsHVDPRev	GGTAAATACCATTGGTCTTGTTCC	~50.000 GU/50µL purified DNA	fresh or frozen mollusc tissue	no	Webb *et al* ., 2007
conventional	apoptosis inhibitor	IA1	AATCCCCATGTTTCTTGCTG	IA2	CGCGGTTCATATCCAAAGTT	not stated	fresh or frozen mollusc tissue	yes	Segarra *et al* ., 2010
conventional	deletion	Del36-37F	ATACGATGCGTCGGTAGAGC	Del36-37R	CGAGAACCCATTCCTGTAA	not stated	fresh or frozen mollusc tissue	yes*	Renault *et a* l., 2012
conventional	unknown protein	CF	CCCCGGGGAAAAAGTATAAA	CR	GTGATGGCTTTGGTCAAGGT	not stated	paraffin-embedded tissues	yes	Renault *et al* ., 2012
nested	unknown protein	A3 A5	GCCAACCGTTGGAACCATAACAAGC CGCCCCAACCACGATTTTTCACTGACCC	A4 A6	GGGAATGAGGTGAACGAAACTATAGACC CCCGTCAGATATAGGATGAGATTTG	500 fg viral DNA/µL TE buffer	fresh or frozen mollusc tissue	no	Renault *et al* ., 2000
real-time	apoptosis inhibitor	B4	ACTGGGATCCGACTGACAAC	B3	GTGGAGGTGGCTGTTGAAAT	not stated	paraffin-embedded tissues	no	Pépin *et al* ., 2008
real-time	unknown protein	C9	GAGGGAAATTTGCGAGAGAA	C10	ATCACCGGCAGACGTAGG	4 GU μL^{-1} purified DNA	paraffin-embedded tissues	no	Pépin *et al* ., 2009
real-time	apoptosis inhibitor	OsHV1BF	GTCGCATCTTTGGATTTAACAA	B4	ACTGGGATCCGACTGACAAC	6 GU mg^{-1} of tissue	fresh or frozen mollusc tissue	no	Martenot *et al* ., 2010
real-time	DNA polymerase	HVDPFor	ATTGATGATGTGGATAATCTGTG	HVDPRev	GGTAAATACCATTGGTCTTGTTCC	~50.000 GU/50µL purified DNA	fresh or frozen mollusc tissue	no	EURL SOP, 2011

Table 3 Polymerase chain reaction methods for the detection of *Ostreid herpesvirus type 1* . TE buffer : 10 mM Tris, 1 mM EDTA, pH 8; *, the presence of the deletion is highly frequent in the µVar genotype but not discriminant in 100% of the cases.

PART II
EXPERIMENTAL APPROACHES

2.1. What is the health status related to OsHV-1 of wild stocks of molluscs along the Italian Mediterranean coast?

In the previous literature review, it has been stated that different bivalve species, the young stages in particular, are susceptible to OsHV-1 infection. Most of them are of commercial interest but it is not excluded that other mollusc species may be susceptible to the disease or play a role as reservoir species. However, in the case of the OsHV-1 microvariants and according to the OIE Aquatic Manual (Version adopted by the World Assembly of Delegates of the OIE in May 2014), the Pacific and the Portuguese cupped oysters are identified as the susceptible species (Arzul *et al.*, 2013), in which the probability of virus detection is higher. Since mollusc farming is an important economic activity in Italy, but also with the aim to prevent a potential contamination of the autochthonous mollusc species that represent an important component of the biodiversity, the attainment of knowledge on the current health status of wild populations of marine molluscs, focusing on cupped oysters and especially in farming areas, is of primary importance.

Thus, during the spring and summer of 2012, 2014, and 2015, 1278 individuals of different marine mollusc species have been collected from natural populations in various locations along the Italian coasts, as reported in Table 4 and Figure 24. Individuals of the different species have been taken from their various natural habitats. After collection, the specimens have been immediately placed in a refrigerated box and processed within maximum 24 hours, as reported in Appendix B.1. All individuals appeared healthy, with no gross lesions.

Fig. 24 Location of the sampling campaigns carried out during 2012, 2014, and 2015.

date	location	number	species	type	latitude	longitude
12 July 2012	Capoiale-Varano**	60	*Crassostrea* sp.	Wild	41°54'22.74"N	15°40'53.75"E
15 July 2012	Fiorenzuola	60	*Crassostrea* sp.	Wild	43°57'16.59"N	12°49'30.84"E
18 July 2012	Chioggia**	60	*Crassostrea* sp.	Wild	45°12'19.24"N	12°14'54.15"E
22 July 2012	Cervia	60	*Crassostrea* sp.	Wild	44°19'55.01"N	12°20'20.08"E
20 May 2014	Caorle	30	*Crassostrea* sp.	Wild	45°35'7.02"N	12°52'11.52"E
20 May 2014	Caorle	4	*Ostrea edulis*	Wild	45°35'7.02"N	12°52'11.52"E
20 May 2014	Monfalcone*	30	*Crassostrea* sp.	Wild	45°46'50.02"N	13°32'22.91"E
20 May 2014	Chioggia**	30	*Crassostrea* sp.	Wild	45°12'19.24"N	12°14'54.15"E
20 May 2014	Muggia*	30	*Crassostrea* sp.	Wild	45°36'32.92"N	13°44'34.79"E
20 May 2014	P. Garibaldi[P]	30	*Crassostrea* sp.	Wild	44°40'35.55"N	12°14'56.88"E
3 June 2014	Caleri**	30	*Crassostrea* sp.	Wild	45° 5'10.20"N	12°19'37.49"E
4 June 2014	Capoiale-Varano**	30	*Crassostrea* sp.	Wild	41°54'22.74"N	15°40'53.75"E
19 June 2014	Cervia	30	*Crassostrea* sp.	Wild	44°19'55.01"N	12°20'20.08"E
19 June 2012	Fiorenzuola	30	*Crassostrea* sp.	Wild	43°57'16.59"N	12°49'30.84"E
26 June 2014	Orbetello**	30	*Crassostrea* sp.	Wild	42°25'55.26"N	11°9'45.50"E
3 July 2014	Marano**	30	*Crassostrea* sp.	Wild	45°45'16.68"N	13°9'51.52"E
23 July 2014	Giulianova[P]	30	*Crassostrea* sp.	Wild	42°45'18.85"N	13°58'37.92"E
10 June 2015	Giulianova[P]	35	*Mytilus* sp.	Wild	42°45'18.85"N	13°58'37.92"E
10 June 2015	Capoiale-Varano**	50	*Mytilus* sp.	Wild	41°54'22.74"N	15°40'53.75"E
11 June 2015	Caleri**	50	*Mytilus* sp.	Wild	45° 5'10.20"N	12°19'37.49"E
11 June 2015	Chioggia**	35	*Mytilus* sp.	Wild	45°12'19.24"N	12°14'54.15"E
24 June 2015	La Spezia	53	*Limaria tuberculata*	Wild	44°04'43.10"N	9°51'45.42"E
24 June 2015	La Spezia	29	*Patella* sp.	Wild	44°04'43.10"N	9°51'45.42"E
24 June 2015	La Spezia	20	*Chlamys varia*	Wild	44°04'43.10"N	9°51'45.42"E
24 June 2015	La Spezia	32	*Anomia ephippium*	Wild	44°04'43.10"N	9°51'45.42"E
24 June 2015	La Spezia	30	*Ostrea edulis*	Wild	44°04'43.10"N	9°51'45.42"E
24 June 2015	La Spezia	50	*Mytilus* sp.	Wild	44°04'43.10"N	9°51'45.42"E
7 July 2015	Muggia	1	*Ostrea edulis*	Wild	45°36'32.92"N	13°44'34.79"E
8 July 2015	Caorle	8	*Chlamys glabra*	Wild	45°35'7.02"N	12°52'11.52"E
8 July 2015	Caorle	2	*Aequipecten opercularis*	Wild	45°35'7.02"N	12°52'11.52"E
8 July 2015	Caorle	18	*Anomia ephippium*	Wild	45°35'7.02"N	12°52'11.52"E
8 July 2015	Caorle	38	*Ostrea edulis*	Wild	45°35'7.02"N	12°52'11.52"E
10 July 2015	Marano	50	*Mytilus* sp.	Wild	45°45'16.68"N	13°9'51.52"E
11 July 2015	Chioggia	50	*Ruditapes philippinarum*	Wild	45°12'19.24"N	12°14'54.15"E
15 July 2015	San Teodoro	50	*Ruditapes decussatus*	Wild	40°48'25.64"N	9°40'05.3"E
15 July 2015	La Spezia	32	*Ostrea edulis*	Wild	44°04'43.10"N	9°51'45.42"E
29 July 2015	La Spezia	20	*Ostrea edulis*	Wild	44°04'43.10"N	9°51'45.42"E
30 July 2015	Caleri	50	*Ruditapes philippinarum*	Wild	45°05'10.20"N	12°19'37.49"E
30 July 2015	Caleri	11	*Parvicardium* sp.	Wild	45°05'10.20"N	12°19'37.49"E
30 July 2015	Caleri	6	*Solen marginatus*	Wild	45°05'10.20"N	12°19'37.49"E

Table 4 Presentation of the 1278 analysed mollusc samples.*, gulf; **, lagoon, [P], harbour.

2.1.1. Which is/are the species of cupped oysters present in the natural beds in Italy?

Several cupped oyster beds are present along the Italian coasts. Individuals can be observed attached mainly on solid surfaces such as rocks and concrete structures that constitute harbours, and also in sheltered areas, such as lagoons, where they can be easily found lying on the sediments. Oysters are particularly numerous in the intertidal zone and in several geographic areas such as Northern and Central Western Adriatic coasts, in particular. None of the different cupped oyster species recorded around the world is originated from Europe, where, if present, they are the result of accidental or deliberate introduction, mainly from East Asia that counts the major number of *Crassostrea* species. This is also the case of

the so called “Portuguese oyster” *C. angulata* that is retained to be present in Europe for at least four centuries, introduced from Asia by merchant ships (O’Foighil *et al*., 1998; Huvet *et al*., 2000b; Batista *et al*., 2005). To date, the exact origin of the Italian populations of cupped oysters has not been clearly established. The first experimental trials of farming in Italy occurred during the late sixties or first seventies, through the introduction of both *C. gigas* and *C. angulata*, and maybe *C. virginica* also (Ghisotti, 1971). However, Ghisotti (1971) reported that in 1969 a cupped oyster bed of 24 km-long was already present in an area situated in the North-Western Adriatic (Sacca degli Scardovari), with a minimum density of 10 individuals per m^2. The presence of important centuries-old commercial harbours and the intensive maritime traffic occurring in the Mediterranean Sea may have influenced the introduction of various species of the genus *Crassostrea*, in the same way of experimental trials, and this introduction may have occurred long time ago. In any case, thanks to optimal environmental conditions, cupped oysters successfully established and spread, and today wild populations are intensively present along the Italian coasts.

On the basis of these considerations, we wanted to be sure to target the two most susceptible species, which are *C. gigas* and *C. angulata* (OIE, 2014), in the following step that aimed to determine the health status related to OsHV-1 of wild mollusc populations. Thus, we determined the species of the cupped oysters collected from the Italian natural populations. The results of the first step of analyses are illustrated in **Publication 1**. In the next paragraphs we will detail the successive steps reached to deepen the characterisation of the Italian populations of cupped oysters.

Sampling

During July 2012 and between May and July 2014, a total of 600 individuals of cupped oysters have been collected from natural beds, as reported in Table 3. Individuals were taken at a depth of around 0.5 m under the low-water line. An uneven presence of natural populations of cupped oyster was observed during this study between Eastern and Western Italian coasts, resulting in 11 sampling sites in Adriatic Sea and only one in Tyrrhenian Sea. The apparent rarity of cupped oysters along the Western Italian coasts, especially in areas where farming is practiced since numerous years such as La Spezia and San Teodoro, may be due to environmental conditions unsuitable for oyster reproduction or larvae settlement. In fact, the salinity exceeds the optimal range for the reproduction of *C. gigas* established by Mann *et al*. (1991).

Taxonomic assignment based on *COI* sequencing

As reported in **Publication 1**, a first taxonomic assignment of specimens was done using a morphology-based approach, with the support of identification keys, as reported by Manzoni (2010). However, it appeared evident that this method was insufficient for a correct taxonomic assignment. Since phenotypic characters are highly plastic within the *Ostreidae* family, depending on environment characteristics and population density, the species identification was then confirmed by molecular methods. Firstly, 25 individuals were subjected to genetic analysis using only the mitochondrial cytochrome c oxidase subunit I gene (*COI*) as genetic marker (Bucklin *et al*., 2011). Yu *et al*. (2003) concluded that *COI* sequence is very sensitive in discriminating closely related *Crassostrea* species, such as *C. gigas* and *C. angulata* and this gene has been used in various studies aiming to *Crassostrea* taxonomic identification and phylogeny (Reece *et al*., 2007; Wu *et al*., 2010). Oyster DNA was extracted as reported in Appendix B.1. A fragment of approximately 655 bp of *COI* was amplified using the primer pair COIfish_F1 (5' TCAACYAATCAYAAAGATATYGGCAC 3') and COIfish_R1 (5' ACTTCYGGGTGRCCRAARAATCA 3') (Ward *et al*., 2005). The Italian samples formed a cluster with the reference sequences of *C. gigas* present in GenBank. On account of the results of this first step of analyses it appears that all the specimens of Italian cupped oyster, subjected to *COI* sequencing, were *C. gigas*.

However, a conspicuous literature has shown that *C. gigas* and *C. angulata* are able to hybridise. The second step of the study was dedicated to verify the hypothesis that Italian oyster populations are the result of hybridisation between these two species.

Frequency of a *C. angulata* allele using a nuclear marker and real-time PCR

Even if the taxonomic status of *C. gigas* and *C. angulata* has been a matter of controversy for years, it is now assumed that they are two different taxa according to genetic evidences based on cytogenetic (Leitão *et al*., 1999) and phylogenetic analyses, in particular based on the mitochondrial *COI* gene (Boudry *et al*., 1998; O'Foighil *et al*., 1998) and nuclear microsatellites (Huvet *et al*., 2000b). Phenotypic differences, represented essentially by superior production yield in the natural environment in France, with a growth factor twice higher in *C. gigas*, have also been reported (Soletchnik *et al*., 2002). This debate was essentially attributable to the hybridisation capacity between the two species (see review of Gaffney and Allen, 1993). In fact, the biological species concept stated by Mayr (1942) considers the reproductive isolation as the main discriminator of species and, on the contrary,

hybrids *C. gigas* x *C. angulata* are viable and also fertile (Menzel, 1974; Huvet *et al*., 2002). The use of a mitochondrial gene in the first step of the study is unsuited to the study of hybrids and hybridisation processes between *C. gigas* x *C. angulata*. Indeed, its haploid nature due to the exclusive maternal inheritance of mtDNA in most animal species (Boursot and Bonhomme, 1986), comprising oysters, precludes the possibility to evidence hybridisation in our samples.

Thus, in this second step, we used a nuclear marker. For this, we developed a real-time PCR focusing on a genome area termed CG44, flanking a microsatellite locus. Huvet *et al*. (2004) evidenced that the majority of *C. gigas* genomes shows a deletion of 21 bp in this region if compared to *C. angulata*, as reported in Figure 25. *Ad hoc* primer pair and TaqMan® probe have been designed to target the deleted region. The primer pair CG44-S (5' ATGATCAACAAATCAACCGT 3') and CG44-AS (5' GTGTATACATGTACATGTAC 3'), and the CG44-probe (5' TTTGGCTTTACCAAATGACAGTTTATAGC 3') were used. The reaction volume of 25 µL contained 12.5 µL of Takara Premix Ex TaqTM 2X (Takara Bio Inc., Shiga, Japan), 0.5 µL of each primer (20 µM), 0.5 µL of CG44-probe (10 µM), 9 µL of DNA/nuclease-free water, and 2 µL of previously extracted DNA. PCR amplification was performed using SmartCycler® (Cepheid®, Sunnyvale, CA, USA) under the following thermal cycling conditions: 1 cycle at 95°C for 10 s, 40 cycles at 95°C for 5 s and at 60°C for 30 s.The positive control consisted in DNA of a *C. angulata* specimen collected in Portugal and extracted as reported in Appendix B.1. A negative control was included and consisted of 2 µL of DNA/nuclease-free water.

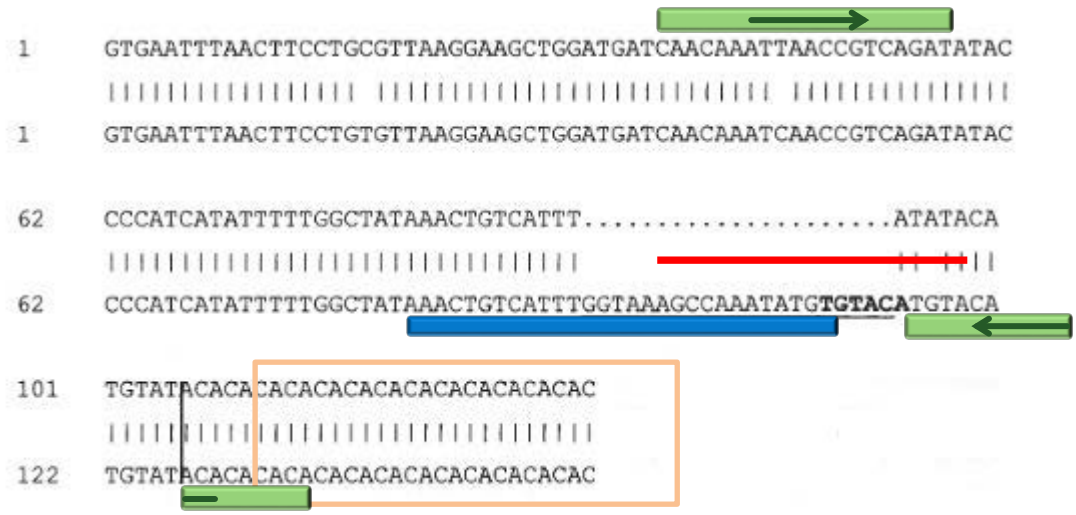


Fig. 25 Alignment of the *C. gigas* (above) and *C. angulata* (below) alleles of the CG44 nuclear marker. Primers: green bars; probe: blue bar. The red line evidences the 21 bp deletion. The microsatellite region is orange-framed. (adapted from Huvet *et al*., 2004)

The molecular analysis on the nuclear marker has been carried out on the DNA extracts (Appendix B.1) of 10 individuals of each of the 12 sampled populations (120 specimens), randomly selected. Twenty-five samples were those already analysed during the first step of *COI* sequencing. The run was considered valid only if the negative controls did not present any amplification, and the Ct value was ≤35. Finally, the genetic analysis, using *COI* as marker, has been extended to the 95 remaining samples. The resultant sequences were subjected to BLAST® 2.6.0 analysis.

The results are presented in Table 5. Amplification of the *COI* gene was unsuccessful for 12 specimens and the sequence was too short to be useful for other 6 individuals. Two of the unsuccessfully amplified samples were positive for *C. angulata* nuclear marker. The mean frequency of the nuclear allele "angulata" in the Italian specimens was 0.25. Its frequency values ranged from 0.0 in the population from Porto Garibaldi to 0.6 in the individuals from Varano. In the other sites, the allele "*angulata*" resulted unequally distributed. The sequencing of the mitochondrial gene confirmed the results obtained during the first step of analyses, and all individuals were identified as *C. gigas* with more than 99% similarities with other *C. gigas* deposited sequences. In Table 6, we reported the frequency of the allele "angulata" for the nuclear marker and of the haplotype "*angulata*" for the mitochondrial marker. These frequencies have been compared to those obtained by Huvet *et al*. (2004) in populations from Japan, Taiwan, France, Portugal, and Spain. In those studies, the deletion associated with the "*gigas*" allele was evidenced through PCR-RFLP method using *bsrGI*, with the loss of the restriction site. Two geographically distinct groups have been highlighted on the basis of the allele frequencies of both *COI* and nuclear markers in oyster populations: "*gigas*"- related markers resulted present with a very high frequency in Japan, the native area of the species, France, and Northern Spain, while "*angulata*"- related markers are very frequent in Taiwan, Portugal, Southern Spain. In the locations where 100% of the individuals possessed a "*gigas*" haplotype with the mitochondrial marker, the allele "*angulata*" in the nuclear marker resulted present in maximum 13.3% of them, with an average of 9.4%. These proportions are consistent with those observed in the Italian populations of Cervia (10%), Caorle (20%), Fiorenzuola (10%), Giulianova (10%), Muggia (20%), Orbetello (20%), and P. Garibaldi (0%). On the contrary, an unexpected proportion of "angulata" allele was observed in the populations of Caleri (30%), Chioggia (50%), Marano (45%), Monfalcone (30%), and Varano (60%). Using a Chi-Squared test it appeared that the "*angulata*" allele frequency in populations from lagoons (Marano, Chioggia, Caleri, Varano, and Orbetello) was higher than

in those from open waters (Muggia, Monfalcone, Caorle, Cervia, and Fiorenzuola) $\chi^2(1, N=100)=5.87$, p=.0153.

Sample id.	rt PCR C_T value	"angulata" NM	Blast *COI*
Portugal	22.64	yes	angulata
Monfalcone2	25.74	yes	gigas
Monfalcone22	-	no	gigas
Monfalcone 8	-	no	gigas
Monfalcone18	-	no	gigas
Monfalcone29	20.98	yes	gigas
Monfalcone12	-	no	gigas
Monfalcone14	-	no	gigas
Monfalcone6	-	no	gigas
Monfalcone10	-	no	gigas
Monfalcone3	21.62	yes	gigas
Chioggia19	-	no	gigas
Chioggia18	22.55	yes	gigas
Chioggia8	23.78	yes	gigas
Chioggia27	26.7	yes	gigas
Chioggia3	-	no	gigas
Chioggia9	-	no	gigas
Chioggia4	-	no	gigas
Chioggia20	23.47	yes	gigas
Chioggia21	-	no	gigas
Chioggia12	21.43	yes	gigas
Caleri19	-	no	gigas
Caleri20	31.39	yes	gigas
Caleri17	21.75	yes	UN
Caleri22	24.42	yes	gigas
Caleri2	-	no	gigas
Caleri18	-	no	UN
Caleri30	-	no	gigas
Caleri8	-	no	gigas
Caleri4	-	no	UN
Caleri1	-	no	gigas
Marano26	22.61	yes	UN
Marano8	-	no	gigas
Marano14	20.37	yes	gigas
Marano15	-	no	gigas
Marano30	-	no	gigas
Marano3	22.21	yes	gigas
Marano11	24.04	yes	gigas
Marano21	-	no	gigas
Marano25	-	no	UN
Marano2	-	no	UN

Table 5 Results of the molecular analyses conducted on the *COI* mitochondrial marker sequence and by real-time PCR on the nuclear marker NM. The C_T values are reported in the second column.

Sample id.	rt PCR C_T value	"angulata" NM	Blast *COI*
Giulianova12	-	no	gigas
Giulianova25	-	no	UN
Giulianova14	-	no	gigas
Giulianova13	-	no	gigas
Giulianova8	-	no	gigas
Giulianova21	-	no	UN
Giulianova9		no	gigas
Giulianova11	-	no	UN
Giulianova1	22.55	yes	gigas
Giulianova22	-	no	gigas
Varano4	19.67	yes	gigas
Varano6	25.65	yes	gigas
Varano18	-	no	UN
Varano17	-	no	gigas
Varano9	-	no	UN
Varano1	33.66	yes	gigas
Varano14	25.9	yes	gigas
Varano11	21.74	yes	gigas
Varano12	19.05	yes	gigas
Varano16	-	no	gigas
Orbetello2	-	no	gigas
Orbetello7	-	no	gigas
Orbetello21	-	no	gigas
Orbetello14	-	no	gigas
Orbetello28	-	no	gigas
Orbetello13	-	no	gigas
Orbetello12	30.45	yes	gigas
Orbetello1	-	no	gigas
Orbetello22	-	no	gigas
Orbetello4	25.96	yes	gigas
Cervia3	-	no	gigas
Cervia 9	-	no	gigas
Cervia24	-	no	gigas
Cervia28	26.45	yes	gigas
Cervia7	-	no	gigas
Cervia16	-	no	gigas
Cervia8	-	no	gigas
Cervia25	-	no	gigas
Cervia18	-	no	gigas
Cervia13	-	no	gigas

Table 5 Results of the molecular analyses conducted on the *COI* mitochondrial marker sequence and by real-time PCR on the nuclear marker NM. The C_T values are reported in the second column.

Sample id.	rt PCR C_T value	"angulata" NM	Blast *COI*
Fiorenzuola28	22.89	yes	gigas
Fiorenzuola11	-	no	gigas
Fiorenzuola14	-	no	UN
Fiorenzuola13	-	no	gigas
Fiorenzuola5	-	no	gigas
Fiorenzuola26	-	no	UN
Fiorenzuola4	-	no	gigas
Fiorenzuola2	-	no	gigas
Fiorenzuola17	-	no	gigas
Fiorenzuola21	-	no	gigas
P.Garibaldi14	-	no	gigas
P.Garibaldi12	-	no	gigas
P.Garibaldi21	-	no	gigas
P.Garibaldi4	-	no	gigas
P.Garibaldi8	-	no	UN
P.Garibaldi25	-	no	gigas
P.Garibaldi30	-	no	UN
P.Garibaldi7	-	no	gigas
P.Garibaldi9	-	no	gigas
P.Garibaldi29	-	no	gigas
Muggia16	-	no	gigas
Muggia3	-	no	gigas
Muggia4	-	no	gigas
Muggia27	25.66	yes	gigas
Muggia24	22.31	yes	gigas
Muggia26	-	no	UN
Muggia12	-	no	gigas
Muggia8	-	no	gigas
Muggia19	-	no	gigas
Muggia6	-	no	gigas
Caorle8	-	no	UN
Caorle11	21.36	yes	gigas
Caorle3	-	no	gigas
Caorle4	-	no	gigas
Caorle27	-	no	gigas
Caorle23	-	no	gigas
Caorle5	-	no	gigas
Caorle1	23.5	yes	gigas
Caorle9	-	no	gigas
Caorle15	-	no	UN

Table 5 Results of the molecular analyses conducted on the *COI* mitochondrial marker sequence and by real-time PCR on the nuclear marker NM. The C_T values are reported in the second column.

Location	frequency NM allele "angulata"	frequency COI haplotype "angulata"
Muggia (Italy)	0.20	0.00
Monfalcone (Italy)	0.30	0.00
Marano (Italy)	0.40	0.00
Caorle (Italy)	0.20	0.00
Chioggia (Italy)	0.50	0.00
Caleri (Italy)	0.30	0.00
P.Garibaldi (Italy)	0.00	0.00
Cervia (Italy)	0.10	0.00
Fiorenzuola (Italy)	0.10	0.00
Giulianova (Italy)	0.10	0.00
Varano (Italy)	0.60	0.00
Orbetello (Italy)	0.20	0.00
Hiroshima (Japan)	0.13	0.00
Tungkang (Taiwan)*	0.93	1.00
Kaohsiung (Taiwan)*	0.88	1.00
Seudre Estuary (France)	0.11	0.00
Arcachon Bay (France)	0.10	0.00
Vieux.Boucau Bay (France)	0.09	0.08
Orio (Spain)	0.02	0.00
Ribadesella (Spain)	0.11	0.05
Vicedo (Spain)	0.11	0.00
Guadatquivir (Spain)*	0.88	1.00
Cadiz (Spain)*	0.89	1.00
Setubal (Portugal)*	0.93	1.00
Rio Mira Estuary (Portugal)*	0.92	1.00
Tavira (Portugal)*	0.78	0.82
Rio Formosa (Portugal)*	0.86	0.88

Table 6 Frequencies of the allele ”angulata” from the nuclear marker NM, and haplotype “angulata” from the mitochondrial marker. *, *C. angulata* populations. Present data and Huvet et al. (2004).

A phylogenetic tree (Figure 26), bringing all the Italian *COI* sequences obtained during the present study has been built using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2015). All the sequences clustered with the *C. gigas* references present in GenBank. However, interestingly, some individuals from Muggia were located in separated branch with a high bootstrap value, 97% for Muggia12.

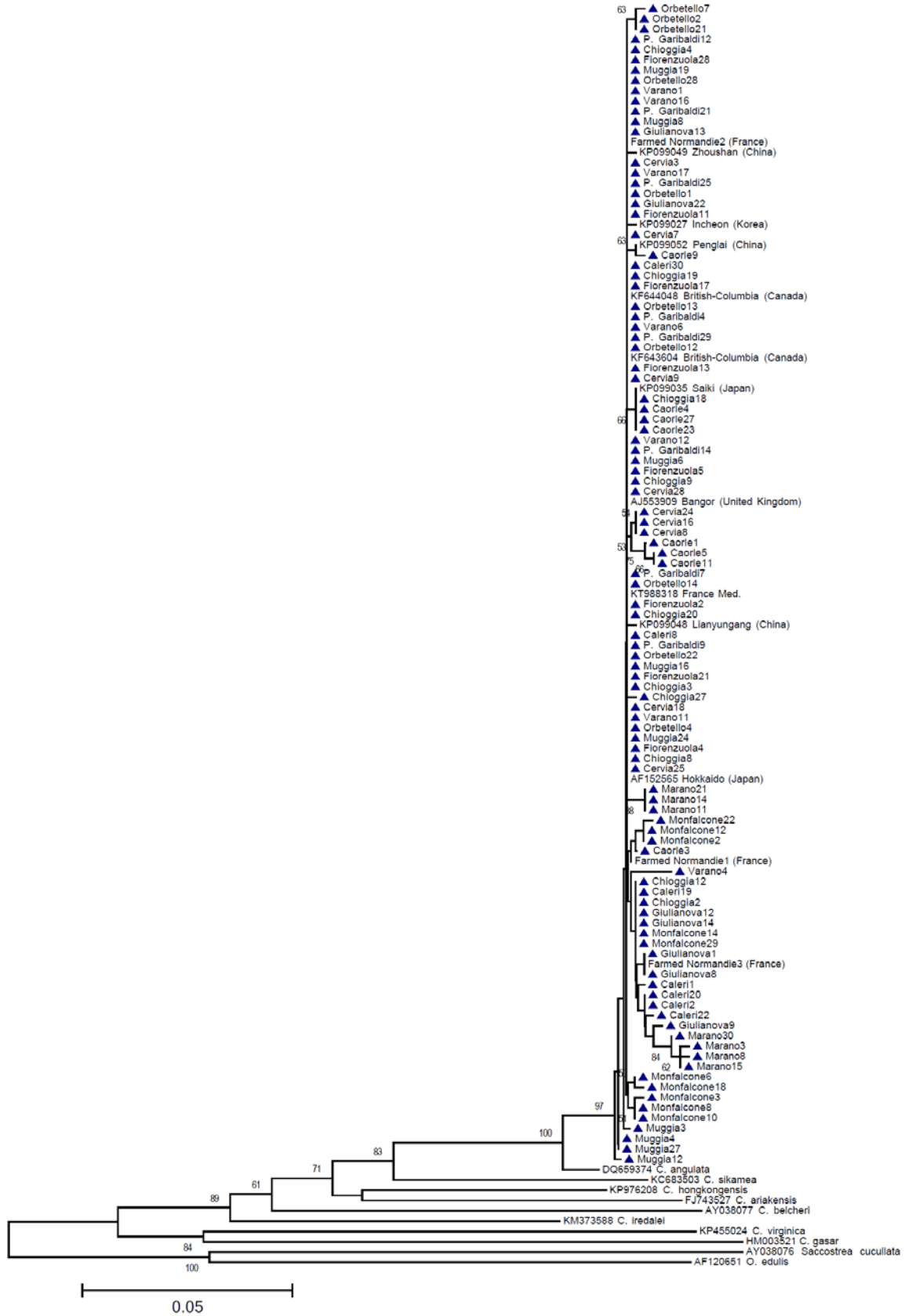


Fig. 26 Neighbor-Joining tree of the cupped oyster individuals, based on *COI* gene analysis. Italian samples are evidenced by a triangle. The bootstrap values (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 123 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 472 positions in the final dataset.

Conclusions

Very little literature is available on the story of the introduction of cupped oysters in Italy, with the purpose to farm them. However, it is presumed that at least two species were introduced quite contemporarily: *C. gigas* and *C. angulata*. In most Italian populations, the frequency of the "angulata" allele is comparable to the frequency observed in consolidated *C. gigas* populations such as in Japan or France. However, the high frequencies observed in some Italian lagoons suggest that hybridisation occurred, even if there is no trace of the mitochondrial "angulata" haplotype in these populations. It would be interesting in future studies to determine also the heterozygosis frequency. At least three hypotheses may be formulated: the Italian wild oyster beds showing high frequency of "angulata" allele are the progeny of a different *C. gigas* population, characterised by a higher frequency of this allele, if compared to the Japanese population at the origin of the French and Spanish *C. gigas* populations; or, the spacial and temporal overlap of the two species *C. gigas* and *C. angulata* during farming trials has been amplified by lagoon conditions, that have slightly balanced the lower fertility ascribed to *C. angulata* (Huvet *et al*., 2002); or, finally, the presence of a point mutation in the restriction site of *BsrGI* in some individuals of *C. angulata* may have underestimated the frequency of this allele in some populations in the study of Huvet *et al*. (2004). Nevertheless, the absence of "angulata" haplotype was not explained.

The present study did not aim to establish the exact origin and phylogeny of the wild oyster populations in Italy but to determine their species. However, these preliminary results showed that Italian individuals may possess interesting genetic characteristics. Thus, Italian populations may be a suitable source of specimens for studies on the selection of zootechnical characters such as growth and especially resistance to pathogens, without the need to import individuals from their native area in East Asia, considering that animal transfers represent a risk of new pathogen introduction.

2.1.2. Determination of the health status of wild Pacific oyster stocks in Italy related to OsHV-1 and genetic polymorphism of the virus.

The aim of this study was to determine the prevalence of OsHV-1 in the wild Italian populations of cupped oysters, which belong to *C. gigas* as shown in the previous chapter, and to identify the viral genotypes present in Italian waters. This work has been published in Journal of Invertebrate Pathology and it is entirely reported at the end of this paragraph as **Publication 1**: **"Ostreid herpesvirus type 1 genomic diversity in wild populations of Pacific oyster *Crassostrea gigas* from Italian coasts". J. Invertebr. Pathol., 137: 71-83.**

To date, most of the studies conducted on OsHV-1 concerned farmed individuals, collected during mortality events. In Europe, little is known about the health status of wild populations even if it is evident that they represent a potential high risk of contamination for farmed stocks. The risk of pathogen diffusion is amplified by the aquatic medium that can easily transport the virus for significant distances.

In a preliminary study, we sampled 240 individuals, divided in two groups on the basis of their length, and consequently on their putative age. These individuals appeared healthy and no mortality was observed during sampling. As in these conditions we expected only low viral loads, we identified the best target organs to use for OsHV-1 detection by real-time PCR to minimize the risk of false-negatives. The labial palps and digestive glands rarely tested positive in infected animals, whereas the heart, muscle, gills, and mantle were proved to be suitable targets. The heart and adductor muscle tested positive in 100% of infected oysters, indicating that they provide the two best target organs in symptom free individuals. Thus, in the successive analyses conducted during our studies, we constituted minced pools of adductor muscle, gill, heart (if the specimen size allowed to), and mantle. The mean prevalence was significantly lower in the small individuals than in the large ones. Although the latency phenomenon has not been demonstrated in OsHV-1 yet, given the characteristics of herpesviruses and the higher probability of exposure to the virus over time, older animals will be more likely infected with OsHV-1, become carriers of the infectious agent, and potential virus releasers during the reactivation phase under conducive conditions.

After this preliminary study that concerned only four sites (Chioggia, Cervia, Fiorenzuola, and Capoiale-Varano), the sampling campaigns have been extended to other eight locations along the Italian coasts, as reported in Figure 24, targeting large individuals.

The global prevalence among the Italian sites was 10.66 %, ranging from 0% 26.6% across all sampling sites. A significant difference between the prevalences in 2012 and 2014 in the same locations has been evidenced and may be imputable to different environmental conditions that influence the virus replication.

Viral genetic polymorphism was investigated by analyzing three regions, ORFs 4/5, ORFs 35/38, and ORFs 42/43. The genome of herpesviruses, as other DNA viruses, is considered quite stable during time, with nucleotidic variations 10,000-fold lower if compared to RNA viruses (Thiry and Thiry, 2011). This difference is due to the exonuclease 3'-5' activity of the DNA polymerase that is able to control and repair mistakes occurred during replication. Nevertheless, we found a certain polymorphism within the OsHV-1 genome (Figure 26): nine genotypes have been identified in 29 infected oysters, including two variants close to the OsHV-1 reference, known since the early 1990s but not reported in European since 2010. Relevant genotype diversity, with geographical distribution of variants, was highlighted in natural environment but a genotype very close to the µVar appeared the most widespread type and resulted present in all the OsHV-1 infected stocks. The low diversity described in the majority of European studies conducted in farmed stocks, may be due to the culture conditions that induce the reduction of the genetic diversity of hosts and viruses alike.
As reported in other studies, the C region was the most variable. The phylogenetic trees corresponding to the three targeted regions and to the concatemers of these sequences showed the insertion of Italian samples within two well-defined clusters: one including the OsHV1-reference, OsHV-1-SB and AVNV, and the other comprising the µVar and only European genotypes, except for two specimens from South Korea, isolated from larvae in hatchery during 2011. Nevertheless, the number of trinucleotide repetitions, three ACT for the µVar (Segarra *et al*., 2010) was found to be insufficient to discriminate cluster membership.

The present study highlighted the need to focus on the determination of the pathogenicity of the different genotypes. Unfortunately, the viral loads observed in animals infected by the divergent Italian variants were too low to carry out experimental infections. Weighty differences have been underlined in the three investigated regions between the reference and microvariant genotypes, but little is known about the rest of the genome. For this reason the complete genome sequencing of the µVar genotype, isolated from individuals sampled during mortality events, appears to be a step of prime importance.

During the present study, the finding of healthy individuals, infected by OsHV-1, µVar-related genotypes as well, could indicate that resistant populations are present in natural environment in Europe, and could be used for genetic selection of broodstocks. However, it is also acceptable that, in natural environment, individuals do not suffer the crowded and stressful rearing conditions that increase their susceptibility to the virus and facilitate its transmission.

Finally, the cluster including OsHV-1 reference, OsHV-1-SB, AVNV, and two Italian genotypes isolated during the present study comprises a large number of OsHV-1 specimens isolated from non-*C. gigas* bivalves. Thus, we can hypothesize that these two Italian variants affect also other mollusc species. We verified this hypothesis in the next study.

Fig. 27 Sequence alignments of the different Italian OsHV-1 genotypes with OsHV-1 reference and OsHV-1 µVar sequences, for the three targeted regions ORFs4/5, ORFs42/43, and ORFs35-38.

Journal of Invertebrate Pathology 137 (2016) 71–83

Contents lists available at ScienceDirect

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Ostreid herpesvirus type 1 genomic diversity in wild populations of Pacific oyster *Crassostrea gigas* from Italian coasts

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ARTICLE INFO

Article history:
Received 23 February 2016
Revised 19 May 2016
Accepted 21 May 2016
Available online 24 May 2016

Keywords:
Ostreid herpesvirus-1
Variants
Crassostrea gigas
Bivalves
Wild population

ABSTRACT

Ostreid herpesvirus 1 (OsHV-1) is a significant pathogen affecting the young Pacific oyster *Crassostrea gigas*, worldwide. A new variant, OsHV-1 µVar, has been associated with recurrent mortality events in Europe since 2008. Epidemiological data collection is key for global risk assessment; however little is known about health status and genotypes present in European wild oyster beds. Most studies to date have involved only cultivated individuals during mortality events, and reported low genotype diversity. With this study, conducted along the Italian coasts, we investigated for the first time the presence of OsHV-1 in European natural oyster beds. Analysis of three genomic regions revealed the presence of at least nine different genotypes, including two variants close to the OsHV-1 reference, known since the early 1990s but with no European record reported since 2010, and highlights relevant genotype diversity in natural environment. Phylogenetic analysis distinguished two distinct clusters and geographical distribution of genotypes, with the exception of a variant very closely related to the µVar, which appeared the single genotype present in all the Adriatic sites. Interestingly, these wild symptom free populations could represent, in Europe, an accessible alternative to the import of OsHV-1-resistant oyster strains from the East Pacific, the native area of *C. gigas*, avoiding the high-risk of non-native marine species and new pathogen introductions.

1. Introduction

Italy ranks third among bivalve mollusc producing countries in Europe (FAO, 2014) with 110.645 t/year. Though clams and mussels still account for the bulk of national production with 31.600 t/year and 79.000 t/year respectively (FAO, 2014), the introduced Pacific cupped oyster, *Crassostrea gigas* (Thunberg, 1793) is becoming an increasingly important product, even if it represents today less than 1% of the total bivalve production and concerns only three regions, Sardinia and, to a lesser extent, Liguria and Veneto. In the past, in Italy, the culture of the native flat oyster *Ostrea edulis* has been performed, since antiquity to the end of the 19th century, before of being almost completely abandoned nowadays. During the seventies, different experimental trials of cupped oyster farming were conducted, through the introduction of both *C. gigas* and *Crassostrea angulata*, but the real producing activities started about ten years ago. Since then, finding optimal environmental conditions, cupped oysters successfully established and spread, and today wild populations are intensively present along the Italian coasts. Actually no commercial hatchery for *C. gigas* is present and the spat collection in open sea is not performed, compelling the producers to import spat from France.

However, the diversification of aquaculture species farmed in Italy and the development of commercial oyster farming raise concern about how to prevent and control the spread of infectious agents. Historically, viral diseases are a recognised cause of disastrous damage in aquatic reared animals, including bivalve molluscs. Between 1967 and 1973, the heavy mortalities of adult oysters due to iridovirus infections (Comps and Duthoit, 1976) decimated Portuguese cupped oyster, *C. angulata* (Lamark, 1819) stocks in France, where they were subsequently replaced by the Pacific cupped oyster introduced from Japan and British Columbia (Mineur et al., 2014). In the past two decades, sporadic summer mortalities in young oysters have occurred in Europe, New Zealand, and Australia (International Ostreid Herpesvirus 1 µVar Workshop, 2011). Since April–May 2008, the intensification of

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http://dx.doi.org/10.1016/j.jip.2016.05.004
0022-2011/

these phenomena was observed, almost simultaneously in all the French production areas, with mortality rates between 60% and 100% (Cochennec-Laureau et al., 2009; Renault et al., 2009). Ostreid herpesvirus 1 (OsHV-1) was considered the aetiological agent responsible for these outbreaks, and the microvariant (μVar) genotype in particular, identified during the massive mortality outbreaks in 2008 (Segarra et al., 2010).

Infection with a herpes-type viruses in invertebrate animals was first reported in 1972 (Farley et al., 1972) and then associated with larvae mortality events in French hatcheries during summer of 1991 (Nicolas et al., 1992) and in the subsequent years (Renault et al., 1994a,b). Analogous events were reported in New Zealand (Hine et al., 1992), California (USA) (Friedman et al., 2005), Mexico (Vasquez-Yeomans et al., 2004), Ireland and Jersey (Peeler et al., 2009), and Australia (Jenkins et al., 2013) and involved larval and juvenile stages. Experimental transmission of the viral disease demonstrated the pathogenicity of OsHV-1, with 100% mortality occurring within 6 days after infection (Le Deuff et al., 1994; Renault et al., 1995). The virus was classified as a member of the order *Herpesvirales* (Davison et al., 2009) and the species termed *Ostreid herpesvirus* (Minson et al., 2000). Belonging to the *Malacoherpesviridae* family, it is the single species of the genus *Ostreavirus* (Davison et al., 2009). The reference sequence of the overall viral genome was first obtained from infected larvae collected in France in 1995 (GenBank AY509253) and its length was estimated at 207 kbp (Davison et al., 2005). The genome organisation, as described by Davison et al. (2005), consisted of two unique regions, UL (167,843 bp) and US (3370 bp), each flanked by inverted repeats TRL/IRL (7584 bp) and TRS/IRS (9774 bp) respectively, and separated by the X region (1510 bp).

Until 2001, only two genotypes were known: the reference type (Davison et al., 2005) and a second genotype termed OsHV-1 Var (Arzul, 2001). Several variants have since then been described by sequencing specific areas of the genome, but essentially within the C region. In 2008, simultaneously with the increase in mass mortality events in oysters, another variant was isolated and called μVar (Segarra et al., 2010). It is formally characterized by a deletion of 12 consecutive bp in the C region (GenBank HQ842610) in a microsatellite locus (EC, 2010; OIE, 2013). Other mutations are present in the μVar genotype: in ORFs 42/43 encoding an apoptosis inhibitor, and in most cases a deletion of 605 bp, corresponding to the total loss of both ORFs 36 and 37 and the partial loss of ORF 38 encoding a RING finger protein (Segarra et al., 2010; Renault et al., 2012). Although the μVar appears to be the most common genotype in C. gigas in France since 2008 (REPAMO, 2010), several other new variants have been described, mainly on the basis of the C region analysis, in France (Martenot et al., 2011, 2012; Renault et al., 2012), Ireland (Lynch et al., 2012; Morrissey et al., 2015), New Zealand (MPI, 2010; Renault et al., 2012), Mexico (Grijalva-Chon et al., 2013), China (Renault et al., 2012), South Korea (Hwang et al., 2013), Australia (Jenkins et al., 2013), and Japan (Shimahara et al., 2012). Retrospective studies have demonstrated that the μVar genotype was already present in 2004 in Normandy (France) (Martenot et al., 2012) and in New Zealand in 2005 (OIE, 2013) but its origin remains uncertain. Related genotypes were described in various bivalve mollusc species in China (Bai et al., 2015; Ren et al., 2013), including the acute viral necrosis virus (AVNV) (GenBank GQ153938) in *Chlamys farreri* and close variants. In this international context, the health status related to OsHV-1 of wild stocks of *C. gigas* along the Italian Mediterranean coast is unknown. In 2010 and 2012, infection with OsHV-1 was described in Italy in young hatchery-reared oysters originating from France, which tested positive for the virus during the first monitoring campaign carried out one month after their introduction in the Adriatic (Dundon et al., 2011; Rosani et al., 2014). As the health status of spat at arrival in Italy is unknown, the question remains whether OsHV-1 was already present in Italian waters. In accordance with European Food Safety Authority recommendations (EFSA, 2015) for the prevention and control of the spread of OsHV-1 and its variants, and given the close contact between wild and reared populations coexisting in the same environment, we conducted this study on wild *C. gigas* populations in Italian coastal waters. For the first time in Europe, to our knowledge, a survey was carried out on natural oyster stocks. As a consequence to the detection of OsHV-1 μVar in Italian farmed stock in the last five years, the potential contamination of surrounding wild beds should be investigated to assess the risk of contamination of virus-free individuals during farming but it is also possible that the virus was introduced with its host during the seventies or was already present in other bivalve species. By testing the presence of OsHV-1 in wild beds and describing these variants, we could obtain precious epidemiological information to answer these questions.

2. Materials and methods

2.1. Sampling

During the spring and summer of 2012 and 2014, a total of 600 individuals of Pacific oyster, *C. gigas* were collected along the Italian coasts. A preliminary study was conducted in June 2012 along the Adriatic where four natural oyster beds (Fig. 1 and Table 1) were investigated to determine the potential presence of OsHV-1 variants. Specimens were taken at a depth of around 0.5 m under the low-water line. To assess the age effect on prevalence, 30 individuals $\leqslant$25 mm in length (small individuals) and 30 individuals $\geqslant$80 mm in length (large individuals) were taken from each site. The specimens were immediately placed in a refrigerated box and processed within 4 h. All individuals appeared healthy, with a good reactivity of the adductor muscle. The small individuals were shucked and deposited in single tubes containing 15 mL Dehyol 70% (Bio-Optica, Milan, Paris) until analysis. The same storage method was used for large individuals, except that the heart, gills, mantle, adductor muscle, labial palps and digestive gland were conserved and processed separately to determine the best target organ for detecting OsHV-1. During a second sampling campaign, carried out between May and June 2014, 30 specimens (>80 mm in length) per site were collected from 12 sampling sites along the Italian coasts. The uneven presence of natural populations of cupped oyster was observed during this study, resulting in 11 sampling sites in Adriatic Sea and only one in Tyrrhenian Sea (Fig. 1 and Table 1). Five sites were located within a lagoon: Marano (Udine), Chioggia (Venezia), Caleri (Rovigo), Capioale (Foggia) and Orbetello (Grosseto), two in a gulf: Muggia (Trieste) and Monfalcone (Gorizia), three in open waters: Caorle (Venezia), Cervia (Ravenna) and Fiorenzuola (Pesaro-Urbino) and two in harbors: Porto Garibaldi (Ferrara) and Giulianova (Teramo). After shell removal and organ separation, pools of tissue from the mantle, gills, heart, and adductor muscle were minced using scalpels, and 2 g of each homogenate were frozen at −20 °C until analysis.

2.2. Oyster species identification

Taxonomic assignment of specimens was done using a morphology-based approach, with the support of identification keys, as reported by Manzoni (2010). However, because phenotypic characters are highly plastic within the Ostreidae family, species identification was confirmed by molecular methods using the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) as the genetic marker (Bucklin et al., 2011). Small samples of mantle tissue, taken from 25 individuals collected from different natural

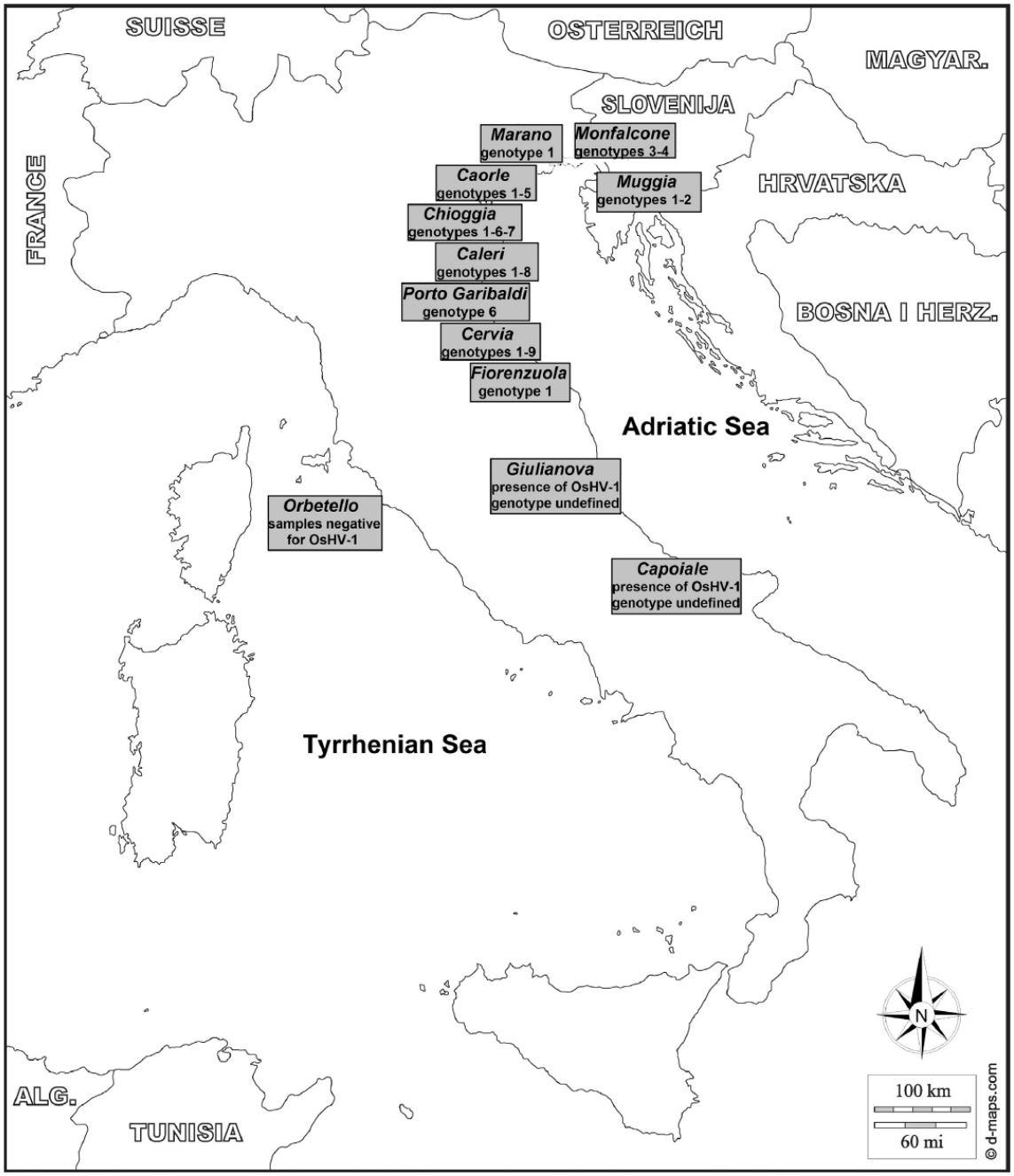

Fig. 1. Location of the sampling campaigns carried out during 2012 and 2014 and geographic distribution of genotypes.

beds situated along the Adriatic were submitted to DNA extraction using a commercial kit (NucleoSpin Tissue, Macherey-Nagel, Oensingen, Switzerland) according to the manufacturer's instructions. A fragment of approximately 655 bp of *COI* was amplified using the primer pair COIfish_F1 (5′ TCAACYAATCAYAAAGATATYGGCAC 3′) and COIfish_R1 (5′ ACTTCYGGGTGRCCRAARAATCA 3′) (Ward et al., 2005) in a volume of 25 µL containing platinum quantitative PCR SuperMix UDG 2X (Invitrogen, Carlsbad, CA, USA), 0.3 µM of each primer, 100 ng of genomic DNA, and ultrapure distilled water to volume. PCR cycling parameters were 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The sequences were determined by direct DNA sequencing on both strands of the PCR products by BigDye Terminator v3.1 cycle sequencing kit (Applied

Table 1
Sampling plan and geographical coordinates of sites.

Sampling site	Latitude	Longitude	Sampling campaign 2012		Sampling campaign 2014	
Muggia	45°36′32.92″N	13°44′34.79″E	–	–	May 20th	30 ind. ⩾ 80 mm
Monfalcone	45°46′50.02″N	13°32′22.91″E	–	–	May 20th	30 ind. ⩾ 80 mm
Marano	45°45′16.68″N	13° 9′51.52″E	–	–	June 26th	30 ind. ⩾ 80 mm
Caorle	45°35′7.02″N	12°52′11.52″E	–	–	May 20th	30 ind. ⩾ 80 mm
Chioggia	45°12′19.24″N	12°14′54.15″E	July 18th	30 ind. ⩽ 25 mm/30 ind. ⩾ 80 mm	May 20th	30 ind. ⩾ 80 mm
Caleri	45° 5′10.20″N	12°19′37.49″E	–	–	June 3rd	30 ind. ⩾ 80 mm
P. Garibaldi	44°40′35.55″N	12°14′56.88″E	–	–	May 20th	30 ind. ⩾ 80 mm
Cervia	44°19′55.01″N	12°20′20.08″E	July 22th	30 ind. ⩽ 25 mm/30 ind. ⩾ 80 mm	June 19th	30 ind. ⩾ 80 mm
Fiorenzuola	43°57′16.59″N	12°49′30.84″E	July 15th	30 ind. ⩽ 25 mm/30 ind. ⩾ 80 mm	June 19th	30 ind. ⩾ 80 mm
Giulianova	42°45′18.85″N	13°58′37.92″E	–	–	June 20th	30 ind. ⩾ 80 mm
Capoiale	41°54′22.74″N	15°40′53.75″E	July 12th	30 ind. ⩽ 25 mm/30 ind. ⩾ 80 mm	June 4th	30 ind. ⩾ 80 mm
Orbetello	42°25′55.26″N	11° 9′45.50″E	–	–	June 10th	30 ind. ⩾ 80 mm

Biosystems, Carlsbad, CA, USA) using the amplification primer pair and analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Sequences were compared with those deposited in the GenBank and BOLD databases. Results were considered valid above 98% similarity. Neighbor-joining trees based on Kimura's 2-parameters distance matrices were built to compare samples with specimens of the phylogenetically closest species. A bootstrap test of confidence was applied to determine the statistical validation of the species assignation (Terol et al., 2002).

2.3. Nucleic acid extraction for OsHV-1 analysis

A mass of 50 mg of minced tissue from each sample was subjected to DNA extraction using a QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol for blood or body fluids, except for elution performed in 60 µL Qiagen elution buffer AE (Martenot et al., 2010). An extraction control was systematically included to prevent false-positives and consisted of 50 µL of DNA/nuclease-free water treated as a sample.

2.4. OsHV-1 detection

OsHV-1 detection was carried out using a real-time PCR protocol based on TaqMan® technology (Applied Biosystems) (Martenot et al., 2010) that targets the B region encoding a putative apoptosis inhibitor (Arzul et al., 2001). The primer pair B3 (5′ GTCGCATCTTTGGATTTAACAA 3′) and B4 (5′ ACTGGGATCCGACTGACAAC 3′), the B3-B4 probe (5′ TGCCCCTGTCATCTTGAGGTATAGA CAATC 3′), and an internal control (IC) probe (5′ ATCGGGGGGGGGGGTTTTTTTTTTATCG 3′) were used. The reaction volume of 25 µL contained 12.5 µL of Takara Premix Ex Taq™ 2X (Takara Bio Inc., Shiga, Japan), 0.5 µL of each primer (20 µM), 0.5 µL of B3-B4 probe (10 µM), 0.5 µL of IC probe (10 µM), 6.5 µL of DNA/nuclease-free water, 2 µL of an IC solution (1.4×10^2 - genome units (GU)/2 µL) consisting of a synthesized sequence containing the complementary sequence of the forward and reverse primers at each end and internally the IC probe sequence, and 2 µL of extracted DNA. PCR amplification was performed in duplicate using SmartCycler® (Cepheid®, Sunnyvale, CA, USA) under the following thermal cycling conditions: 1 cycle at 95 °C for 10 s, 40 cycles at 95 °C for 5 s and at 60 °C for 20 s. A negative control was included and consisted of 2 µL of DNA/nuclease-free water in 23 µL of real-time PCR mix. The extraction control was treated as a sample. To obtain the standard curve different dilutions of plasmidic DNA solution (produced according an internal accredited method), corresponding to the OsHV-1 target region, were used from 10 to 10^5 copies of OsHV-1 DNA units/µL and were also used as positive control. The run was considered valid only if the extraction and the negative controls did not present any amplification. A sample was considered positive only if the difference between the Ct value of the duplicates did not exceed 0.5, the regression coefficient of the standard curve was at least 0.98 and the slope between −4.115 and −2.839, and the Ct value was ⩽38.5. A sample was considered a true negative only if amplification of the IC was successful. After referring to the standard curve, the viral loads were expressed in genome units GU/50 mg of oyster tissue.

2.5. Statistical analysis of prevalence

For the statistical comparisons, the occurrence of the viral agent in oysters was described in terms of prevalence, defined as the number of oysters positive for OsHV-1 in 100 individuals investigated by real-time PCR, associated with the 95% confidence interval (95% CI). Multiple logistic regression was used to test the association between OsHV-1 in oysters and potential risk factors, including host size (length ⩽25 mm or ⩾80 mm), type of marine environment (open waters, gulf, lagoon, harbor) from which oysters were taken, and sampling year (2012 vs. 2014). The association was evaluated by calculating the odds ratio (OR). Statistical analysis was performed using Stata14.1 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, USA, StataCorp LP).

2.6. Variant characterization

To define the OsHV-1 genotype(s) present in Italian waters, three target regions were investigated by three conventional PCRs applied to samples with a sufficient viral load, followed by amplicon sequencing: the C region including ORFs 4/5 (base 178,181–178,889), situated in the inverted repeat region and encoding two proteins of unknown function; the IAP region containing ORFs 42/43 (base 59,949–60,557) encoding an apoptosis inhibitor protein; and a longer region encompassing ORFs 35–38 (base 51,736–53,324) encoding, among others, a putative membrane, a DUT family, and a RING finger proteins (Davison et al., 2005). The protocol was applied to each sample that tested positive for OsHV-1 by real-time PCR (Segarra et al., 2010; Renault et al., 2012). The sequences of the different primer pairs are reported in Table 2 (Renault and Arzul, 2001; Segarra et al., 2010). In case of failure of C region amplification, alternative internal primers called C′F/C′R were used. The PCR reaction volume of 50 µL was the same for each primer pair and consisted of 25 µL of 2x QIAGEN® Multiplex PCR Pre Mix (Qiagen), 1 µL of each primer (20 µM), 19 µL of DNA/nuclease-free water, and 4 µL of extracted DNA eluate. PCR conditions were: activation/initial denaturation at 95 °C for 15 min; 40 amplification cycles at 95 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s, and one final extension cycle at 72 °C for 3 min. The amplicon lengths are presented in Table 2. For each run, a negative control consisting of 4 µL of DNA/nuclease-free water in 46 µL of PCR mix was included. To verify

Table 2
Primers used for genotype characterization of the three target regions ORFs 4/5, ORFs 42/43, and ORFs 35/36/37/38, and the expected amplicon lengths for the OsHV-1 reference genotype.

Description	Target region	Sequences	Amplicon length (bp)	References
C2 (forward)	ORFs 4/5	5′ CTCTTTACCATGAAGATACCCACC 3′	709	Arzul (2001)
C6 (reverse)		5′ GTGCACGGATTACCATTTTT 3′		
C′F (forward)	ORFs 4/5	5′ CCCCGGGGAAAAAAGTATAAATAG 3′	1174	–
C′R (reverse)		5′ CCTCTTCATTGGGATATCAC 3′		
IA1 (forward)	ORFs 42/43	5′ CGCGGTTCATATCCAAAGTT 3′	607	Segarra et al. (2010)
IA2 (reverse)		5′ AATCCCCATGTTTCTTGCTG 3′		
Del35.38-forward	ORFs 35/36/37/38	5′ ATACGATGCGTCGGTAGAGC 3′	989	Renault et al. (2012)
Del35.38-reverse		5′ TTACAGGAATGGGGTTCTCG 3′		

amplification success and correct fragment length, the PCR products were subjected to QIAxcel® system analysis (Qiagen). With the ORF 35/38 primer pair, several specimens from the Cervia sampling site showed two different bands that were separated on 1% agarose gel and purified with a QIAquick® Gel Extraction kit (Qiagen). Amplicons of expected length were sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing both the sense and the antisense strands. DNA sequencing accuracy was measured by the Phred quality score (Q score) and only scores above 30 were considered. The consensus sequence was determined by the alignment with ClustalW 1.81 (http://www.genome.jp/tools/clustalw/) and the comparison of both strand sequences. Our sequences were aligned with the reference OsHV-1 (GenBank accession number AY509253) complete genome and with the µVar sequences of the three regions of interest: C2/C6 (GenBank HQ842610), IAP (GenBank JN800196), and ORFs 35/38 (GenBank JN800248). For each marker, sequences presenting mutations respect of the above references were labeled starting from variant A to variant L. The genotypes were then characterized by assembling together the three genomic markers as they were found in the infected oyster.

In case of absence of amplification of all three regions and in order to exclude false-positive results with the TaqMan® method, the sample was retested in duplicate by an alternative protocol based on SYBR® Green chemistry as reported in the Standard Operating Procedures of the European Union Reference Laboratory for Molluscs Diseases (IFREMER, La Tremblade, France). The primer pair HVDP-F (5′ ATTGATGATGTGGATAATCTGTG 3′) and HVDP-R (5′ GGTAAATACCATTGGTCTTGTTCC 3′) (Webb et al., 2007) amplifies a PCR product of 197 bp and targets ORF 100, which encodes a putative DNA polymerase δ catalytic subunit. Real-time PCR mix consisted of 12.5 µL of premix 2X SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 2.5 µL of each primer (5 µM), 2.5 µL of DNA/nuclease-free water, and 5 µL of the extracted DNA (10 ng/µL). A negative control was included and consisted of 5 µL of DNA/nuclease-free water in 20 µL of real-time PCR mix. Amplification was performed using CFX96 Touch™ (Bio-Rad) as follows: polymerase activation/initial denaturation at 95 °C for 2 min; 40 amplification cycles at 98 °C for 5 s, 60 °C for 5 s, and 72 °C for 45 s; the melting temperature (Tm) curve was acquired using 0.5 °C steps of 5 s from 65 °C to 95 °C.

2.7. Phylogenetic analysis

Phylogenetic analysis was conducted on ORFs 4/5, ORFs 35–38, and ORFs 42/43 individually and on the concatemers of these three sequences to maximise variability. Sequence similarities between the coding region of the isolated genotypes, the OsHV-1 reference, and the µVar were calculated using ClustalW 1.81 (http://www.genome.jp/tools/clustalw/). The phylogenetic trees were then constructed including the DNA sequences of OsHV-1 (all variants) retrieved from GenBank in *C. gigas* and other marine bivalves (Davison et al., 2005; Friedman et al., 2005; Grijalva-Chon et al., 2013; Hwang et al., 2013; Jenkins et al., 2013; Martenot et al., 2011, 2013, 2015; Renault et al., 2012; Segarra et al., 2010; Shimahara et al., 2012; Xia et al., 2015) and of AVNV (GenBank GQ153939) (Ren et al., 2013). 91 sequences of the C region were downloaded from GenBank, but the corresponding ORFs 42/43 and ORFs 35–38 sequences, isolated from the same specimens, were available for only 30 of them. Since the presence of a large indel in the ORFs 35/36/37/38, variations in the number of trinucleotides in the C region, and the inclusion of these gaps in phylogenetic analysis may all affect the result, the analysis was inferred in parallel with the pairwise-deletion option and the complete-deletion option using the neighbor-joining method (Saitou and Nei, 1987) and with a bootstrap test (1000 replicates) using the MEGA version 5 program (Tamura et al., 2011). Evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993).

3. Results

3.1. Identification of oyster species

COI sequences were obtained for each of the 25 samples, all of which belonged to *C. gigas* and showed significant value of similarity. No differences were found between the results achieved in the GenBank and BOLD databases. Three sequences in the phylogenetic tree were excluded because they were too short and their weight would have been irrelevant to the statistical analysis. The remaining 22 samples formed a cluster with the reference sequences of *C. gigas* present in GenBank: at the node, a bootstrap value >70% corresponds to a probability >95% that the cluster is real, so the species assignment was correct. Sequences from individuals belonging to different species of the Ostreidae family led to different clusters with high bootstrap values.

3.2. Real-time PCR results

Prevalence was calculated separately for each group of individuals (large, small, 2012 and 2014) (Table 3). In order to correctly compare the sampling year, the prevalence of OsHV-1 infection in large individuals (length ⩾80 mm) in 2014 was also calculated taking into account only specimens from the 4 sites sampled in 2012 (old sites). The individuals, with at least one virus-positive organ were defined as being positive specimens. The confidence intervals (95% CI) showed a marked difference in virus prevalence between two groups of organs (Fig. 2): in the OsHV-1 infected specimens, the percentage of virus-positivity in both the digestive gland and labial palps was only 12.5% (95% CI 2.6–32.4%) (3/24) and far less than that detected in the heart 75.0% (95% CI 53.3–90.2%) (18/24), adductor muscle 62.5% (95% CI 40.6–81.2%) (15/24), gills 54.2% (95% CI 37.8–74.5%) (13/24), and mantle 50.0% (95% CI 29.1–70.9%) (12/24). Using the target organs with lowest virus-positivity as reference to estimate the association between virus presence and target organ, univariate analysis

Table 3
Prevalences of OsHV-1 (all genotypes) in the different sites and years of sampling.

Sampling site	Juveniles 2012			Adults 2012			Adults 2014		
	Number	Positive	Prevalence %	Number	Positive	Prevalence %	Number	Positive	Prevalence %
Muggia	–	–	–	–	–	–	30	2	6.7
Monfalcone	–	–	–	–	–	–	30	5	16.7
Marano	–	–	–	–	–	–	30	2	6.7
Caorle	–	–	–	–	–	–	30	4	13.3
Chioggia	30	5	16.7	30	6	20.0	30	1	3.3
Caleri	–	–	–	–	–	–	30	3	10.0
P. Garibaldi	–	–	–	–	–	–	30	1	3.3
Cervia	30	5	16.7	30	5	16.7	30	0	0.0
Fiorenzuola	30	0	0.0	30	5	16.7	30	0	0.0
Giulianova	–	–	–	–	–	–	30	4	13.3
Capoiale	30	2	6.7	30	8	26.7	30	6	20.0
Orbetello	–	–	–	–	–	–	30	0	0.0
Total	120	12	10.0	120	24	20.0	360	28	7.8

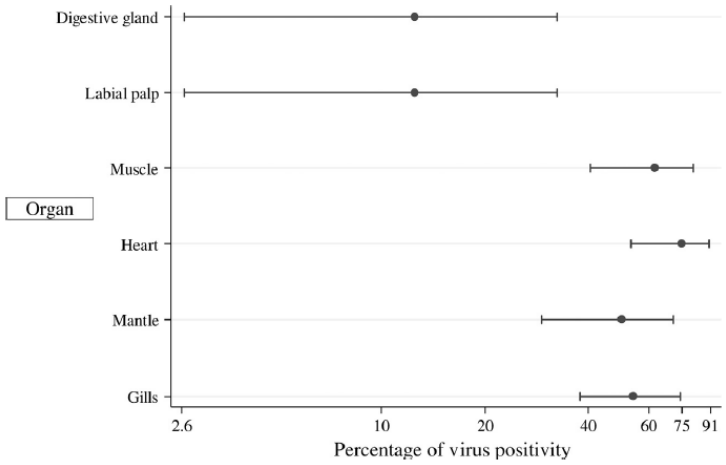

Fig. 2. Percentage of virus positivity by organ (x-axis of this graph), with 95% confidence intervals, in OsHV-1 (all genotypes) infected large individuals in 2012.

showed a significantly lower probability to detect the presence of the virus in the digestive gland and labial palps and a higher probability in the heart (OR 21.0; 95% CI 4.6–96.2), adductor muscle (OR 11.7; 95% CI 2.7–50.5), gills (OR 8.3; 95% CI 1.9–35.3), and mantle (OR 7.0; 95% CI 1.6–29.9). The heart and/or adductor muscle tested positive in 100% of the infected animals, indicating that they are the best targets to detect infection. Only one organ/tissue tested positive in 5/24 individuals (20.8%), 2 organs/tissues in 10/24 (41.7%), 3 organs/tissues in 2/24 (8.3%), 4 organs/tissues in 4/24 (16.7%), 5 in none (0%), and all organs/tissues tested positive in 3/24 (12.5%), all from the Capoiale sampling site. Three or fewer organs tested positive in 70.8% of infected individuals. In the specimens collected during 2012, the prevalence of virus infection was 10% (95% CI 5.3–16.8%) in individuals $\leqslant$25 mm and 20% (95% CI 13.3–28.3%) in individuals $\geqslant$80 mm (Fig. 3). The virus prevalence in the group of large individuals collected in 2014 was 5.8% (95% CI 2.4–11.6%) if we considered only those from the same four sites sampled in 2012 (old sites) and 7.8% (95% CI 5.2–11.0%) when all 12 sites were considered together. The prevalence rates of each sampling site are presented in Table 3. As observed in 2012, prevalence was highest in Capoiale and lowest in Cervia and Fiorenzuola. Comparison by type of environment showed no significant differences between open waters, gulfs, lagoons and harbors. Odds ratio analysis showed a statistical significance for the protective effect of sampling year (2014 versus 2012: OR 0.28; 95% CI 0.12–0.66; $P > 0.004$) and length (small versus large individuals: OR 0.44; 95% CI 0.21–0.93; $P > 0.003$). The viral load in all samples was quite low and ranged from 2.29×10^2 to 3.21×10^5 GU/50 mg in the small individuals and from 2.91×10^2 to 4.71×10^5 GU/50 mg in the large ones. These values were below or just above the viral load threshold of 4.4×10^5 GU/50 mg, beyond which mortality can occur (Oden et al., 2011).

3.3. Sequencing results

A total of 48 positive samples were chosen, on the basis of real-time PCR results, for their sufficient viral load and submitted to conventional PCRs to amplify the three target regions, ORFs 4/5, ORFs 35/38, and ORFs 42/43, followed by sequencing. Amplification was obtained for at least one region in more than 72% of these samples (35/48). However, it was achieved for all the three target regions only in 60.4% of them (29/48) (Table 4). Between the thirteen specimens with no amplification, the samples from Monfalcone (one), Chioggia (four), Cervia (one) and Capoiale (four) have shown a low viral load with real-time PCR, while in the three samples from Giulianova, the viral load was $>5 \cdot 10^3$ GU/50 mg suggesting the presence of amplification inhibitors. With the C2/C6 primer pair, fragments of the expected length of about 700 bp were obtained from 28 samples but amplicon length exceeded 1400 bp in the unique successfully amplified specimen from Giulianova.

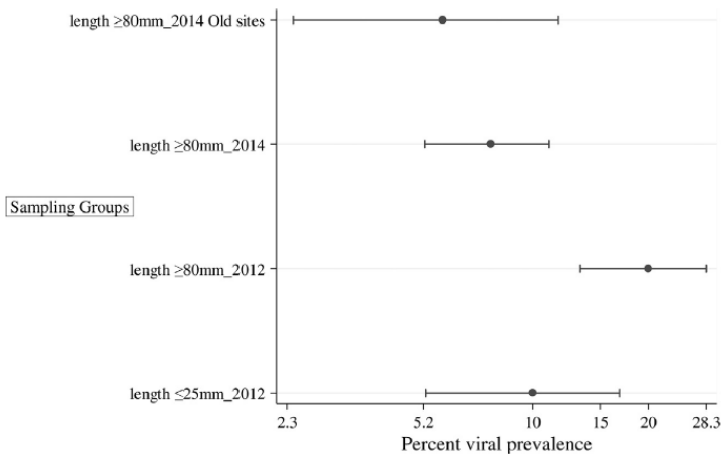

Fig. 3. Prevalence (x-axis of this graph) of OsHV-1 (all genotypes) in the different sampling groups (allocated by length and sampling year), with 95% confidence interval.

Neither the IA1/IA2 nor the Del35.38-F/Del35.38-R primers gave a positive outcome in any of the samples from this location. No amplification of the C region was achieved in three of the four samples from Caorle and in three of the six samples from Cervia using the C2/C6 primer pair. Other forward primers targeting areas upstream of C2 were also unsuccessfully tested. However, a shorter sequence enclosing the variable region of ORFs 4/5 was obtained from these samples when the C2/C6 primer pair was replaced with the CF/CR pair. The ORFs 42/43 and ORFs 35/36/37/38 regions were greatly amplified and gave fragments of expected length. One sample from Cervia and the single sample from Fiorenzuola showed two bands with the Del35.38-F/Del35.38-R primers: a long fragment of around 1000 bp and a shorter one of about 400 bp. These amplification products were separated by electrophoresis, but only the shorter fragments gave a positive response to sequencing.

The three regions showed an interesting genotypic variability. The variants of each marker, with their GenBank references and their origin are reported in Table 4.

The three regions were combined to describe the different genotypes. Samples from specimens with a suspected polyinfection with different virus genotypes, i.e., those presenting a double band with the Del35.38-F/Del35.38-R primers or generating the slightest doubt about the accuracy of the chromatogram result, were excluded. This approach revealed the presence of at least nine genotypes in the natural oyster beds (Table 4). Genotype 1, very close to the μVar, was found in all sites except Monfalcone and was the most frequent one. A geographical distribution of variants was clearly highlighted (Fig. 1).

The two options for handling gaps produced the same distribution of genotypes within the trees but with different phylogenetic distances between them. The phylogenetic trees corresponding to the three targeted regions (C region in Fig. 4) and to the concatemers (Fig. 5) showed the insertion of Italian samples within two well-defined clusters in all cases: one including the OsHV1-reference, OsHV-1-SB and AVNV, and the other comprising the μVar. A total of 100 sequences recorded worldwide were used for the analysis of the C region, including 24 specimens recorded in bivalve species other than *C. gigas*. The sequences issued from French larvae or juveniles <1 year old collected during mortality events before 2008 (Renault et al., 2012), and genotypes 5 from Caorle and 9 from Cervia, isolated during the present study, consistently clustered with OsHV-1 reference/OsHV-1-SB/AVNV. 87.5% of the specimens isolated from non-*C. gigas* bivalves were also included within this group. Interestingly, genotypes 5 and 9 resulted however well separated from the other genotypes within this cluster. In the same way, the remaining Italian genotypes were all assimilated to the μVar cluster. This group comprised only European genotypes, except two specimens from South Korea, isolated from larvae in hatchery during 2011.

Unfortunately, fewer sequences were available for the other two markers. However, a subgroup composed of Italian genotypes 5 and 9 was clearly evidenced for ORFs 42/43, ORFs 35/36/37/39 regions and concatemers (Fig. 5). Genotypes 3 and 4 from Monfalcone constituted another subgroup during the analysis of ORFs 42/43 and concatemers. All samples from France and New Zealand collected after 2008 and most of the Italian samples appeared included in the μVar cluster in the present phylogenetic study.

The results of phylogenetic analysis carried out on the basis of the number of trinucleotide repetitions in the microsatellite region alone were not in line with the classification obtained with the three previous analyses. For instance, the AVNV was included in the μVar cluster. The degree of similarity between the isolated sequences, the OsHV-1 reference, and the μVar was evidenced through the analysis of nucleotide homology in coding regions. The percentage of homologies between the OsHV-1 reference and OsHV-1 μVar sequences was lower than 68% as it is heavily influenced by the presence of indels. Homology calculated within the two groups corresponding to the two clusters was: 97.49–96.55% (ORFs 4/5), 99.65% (ORFs 42/43), and 98.34% (ORFs 35/36/37/38) between genotypes 5 and 9 and the OsHV-1 reference, respectively; and 99.68–100% (ORFs 4/5), 99.82–100% (ORFs 42/43), and 99.32–99.66% (ORFs 35/36/37/38) between genotype 1, 2, 3, 4, 6, 7, and 8, and the OsHV-1 μVar, respectively.

4. Discussion

Ostreid Herpesvirus 1 microvariants (μVar and related genotypes presenting sequence variations in the microsatellite locus upstream of ORF 4) are considered emergent viral pathogens for young stages of cupped oysters *C. gigas* and *C. angulata* (OIE, 2013) and believed to be responsible for the increase in mass mortality events observed worldwide since 2008. Finding effective measures to prevent and control disease has remained elusive due in part to mounting evidence of variants with unknown pathogenicity and scarce information on the health status of wild oyster populations in Europe. Oyster farming is still underdevel-

Table 4
Variants by marker (ORFS 42/43; ORFS 35/38; ORFS 4/5), with the corresponding GenBank accession numbers, and genotypes detected in oysters from wild beds in Italy.

		Variants identified by marker							Genotypes
		ORFs42/43	Id. GenBank	ORFs35/38	Id. GenBank	ORFs4/5		Id. GenBank	
						C2/C6	CF/CR		
Sampling sites									
Muggia	Specimen 1	µVar type		Variant F	KT954024	µVar type		KT954011	Genotype 1
	Specimen 2	µVar type		Variant F		Variant G			Genotype 2
Monfalcone	Specimen 1	Variant A	KT954018	Variant F	KT954024	µVar type			Genotype 3
	Specimen 2	Variant A		Variant F		µVar type			Genotype 3
	Specimen 3	Variant A		Variant C	KT954021	Variant H		KT954012	Genotype 4
	Specimen 4	Variant A		Variant C		Variant H			Genotype 4
	Specimen 5	–		–		–	–		–
Marano	Specimen 1	µVar type		Variant F	KT954024	µVar type			Genotype 1
	Specimen 2	µVar type		Variant F		µVar type			Genotype 1
Caorle	Specimen 1	µVar type		Variant F	KT954024	µVar type			Genotype 1
	Specimen 2	Variant B	KT954019	Variant D	KT954022	–	Variant L	KT954017	Genotype 5
	Specimen 3	Variant B		Variant D		–	Variant L		Genotype 5
	Specimen 4	Variant B		Variant D		–	Variant L		Genotype 5
Chioggia	Specimen 1	µVar type		Variant F	KT954024	µVar type			Genotype 1
	Specimen 2	µVar type		Variant F		µVar type			Genotype 1
	Specimen 3	µVar type		Variant E	KT954023	µVar type			Genotype 6
	Specimen 4	µVar type		Variant E		Variant I		KT954013	Genotype 7
	Specimen 5	µVar type		Variant E		Variant I			Genotype 7
	Specimen 6	–		–		–	–		–
	Specimen 7	–		–		–	–		–
	Specimen 8	–		–		–	–		–
	Specimen 9	–		–		–	–		–
Caleri	Specimen 1	µVar type		Variant F	KT954024	µVar type			Genotype 1
	Specimen 2	µVar type		Variant F		Variant J		KT954014	Genotype 8
	Specimen 3	µVar type		Variant F		Variant J			Genotype 8
P. Garibaldi	Specimen 1	µVar type		Variant E	KT954023	µVar type			Genotype 6
Cervia	Specimen 1	µVar type		Variant F	KT954024	µVar type			Genotype 1
	Specimen 2	µVar type		Variant F		µVar type			Genotype 1
	Specimen 3	µVar type		Variant F		µVar type			Genotype 1
	Specimen 4	Variant B	KT954019	Variant D	KT954022	–	Variant K	KT954016	Genotype 9
	Specimen 5	Variant B		Variant D		–	Variant K		Genotype 9
	Specimen 6	Variant B		Variant D		–	Variant K		Genotype 9
	Specimen 7	–		–		–	–		–
Fiorenzuola	Specimen 1	µVar type		Variant F	KT954024	µVar type			Genotype 1
Giulianova	Specimen 1	–		–		Aspecific	–		–
	Specimen 2	–		–		–	–		–
	Specimen 3	–		–		–	–		–
	Specimen 4	–		–		–			–
Capoiale	Specimen 1	µVar type		Aspecific		µVar type			–
	Specimen 2	µVar type		Aspecific		µVar type			–
	Specimen 3	µVar type		Aspecific		µVar type			–
	Specimen 4	µVar type		Aspecific		µVar type			–
	Specimen 5	µVar type		Aspecific		µVar type			–
	Specimen 6	–		–		–	–		–
	Specimen 7	–		–		–	–		–
	Specimen 8	–		–		–	–		–
	Specimen 9	–		–		–	–		–

oped in Italy, except for three regions in Sardinia and, to a lesser extent, Liguria and Veneto. Following experimental trials conducted during the late 1970s in areas along the Adriatic and Tyrrhenian coasts, various different species, essentially *C. gigas* and *C. angulata*, phylogenetically very close and some capable of hybridization, were introduced. In this survey, we used the mitochondrial *COI* gene to identify oyster species composing the natural Italian populations of cupped oysters. The results permitted us to exclude the presence of species other than *C. gigas* and *C. angulata* but not to individually discriminate any hybrid *C. gigas* × *C. angulata*. Because all individuals were identified as *C. gigas* in an established natural population, we assumed that pure individuals of *C. angulata* would be present only in very few numbers or even absent in the sampled areas. Both species are susceptible to infection with OsHV-1.

In the present work, for the first time in Europe, we focused the study of OsHV-1 on wild stocks of *C. gigas*, others being in East Asia. No ongoing mortality was observed and all specimens seemed healthy.

The real-time PCR method has high sensitivity (detection limit 6 GU/mg of tissues) for detecting OsHV-1 (Martenot et al., 2010). Furthermore, to minimize the risk of false-negatives in infected adult animals, we identified the best target organ for diagnostic testing and separately analyzed the organ/tissues isolated from individuals sampled in 2012. A significant difference in virus positivity was found between two groups of organs: the labial palps and digestive glands rarely tested positive in infected animals, whereas the heart, muscle, gills, and mantle proved suitable targets. The heart and adductor muscle tested positive in 100% of infected oysters, indicating that they provide the two best target

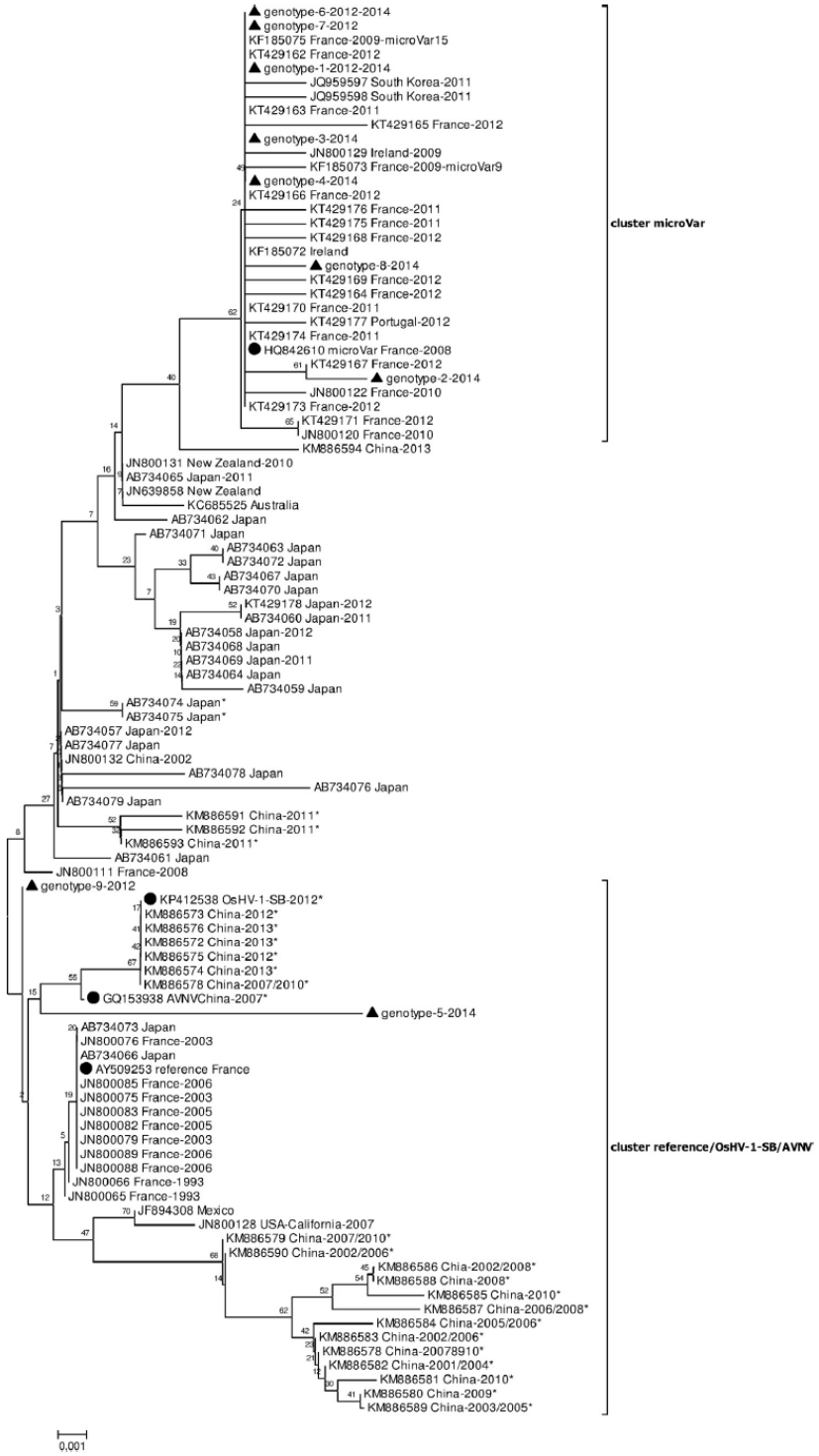

Fig. 4. Neighbor-joining tree (pairwise-deletion option) of the C region. The phylogenetic analysis includes the sequences of the OsHV-1 reference (●), OsHV-1 μVar (●), AVNV (●), and OsHV-1 sequences retrieved from GenBank and the Italian isolates (▲). Bootstrap values are shown above the internal node. The tree is drawn to scale to represent the evolutionary distances between isolates.

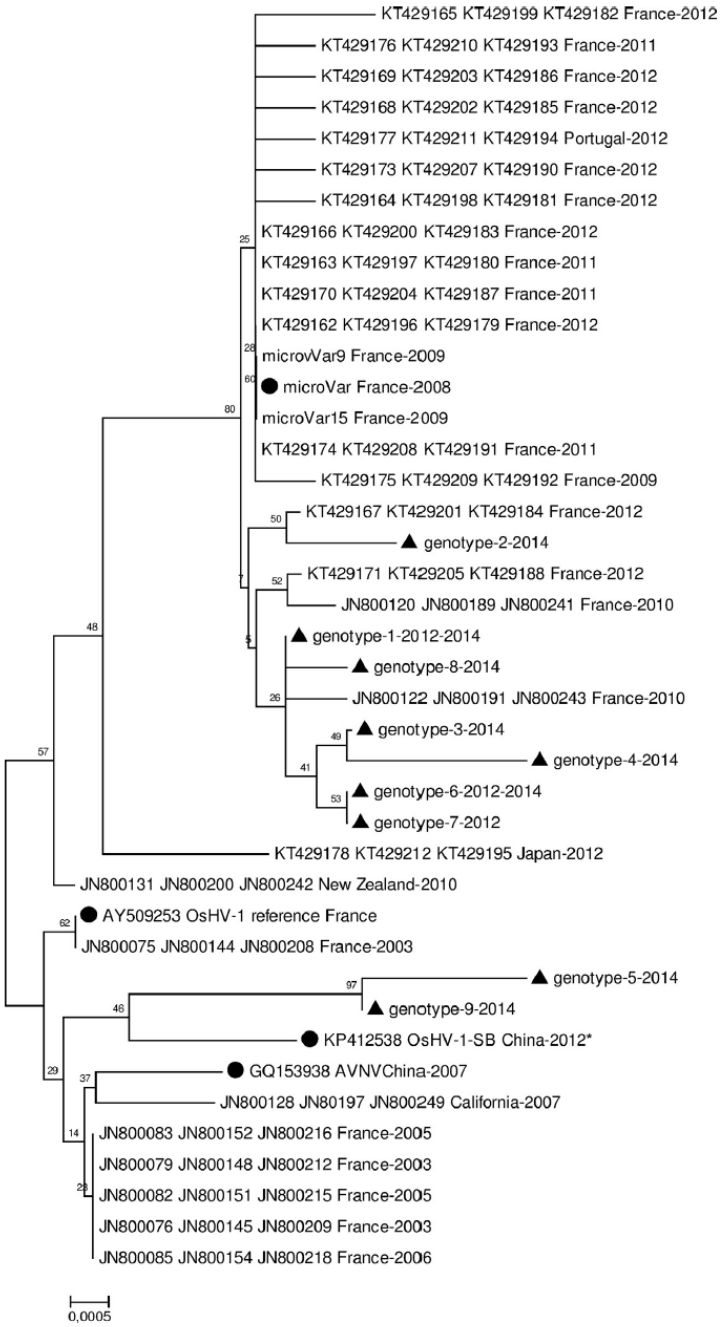

Fig. 5. Neighbor-joining tree (pairwise-deletion option) of the concatemers. The phylogenetic analysis includes the sequences of the OsHV-1 reference (●), OsHV-1 µVar (●), AVNV (●), and OsHV-1 sequences retrieved from GenBank and the Italian isolates (▲). Bootstrap values are shown above the internal node. The tree is drawn to scale to represent the evolutionary distances between isolates.

organs. However, because acquiring heart in smaller animals is quite difficult, an easier, more accurate method is to pool tissues from the adductor muscle, gills, mantle, and heart together when possible.

OsHV-1 prevalence in wild oysters was quite low and ranged from 0% to 26.7% across all sampling sites. The mean prevalence was significantly lower in the small than in the large individuals. Although the latency phenomenon has not yet been demonstrated in OsHV-1, given the characteristics of herpesviruses and the higher probability of exposure to the virus over time, older animals will more likely be infected with OsHV-1, become carriers of the infectious agent, and potential virus releasers during the reactiva-

tion phase under conducive conditions. Virus prevalence was markedly lower in 2014 as compared with 2012 across all sampling sites. A possible explanation for the difference is that, because the environmental conditions in 2014 were less conducive to virus replication than in 2012, the viral loads were under the detection limit of the diagnostic method or even absent. Of note, however, is that we did not investigate the effect of different storage methods (Dehyol and freezing) used in the two sampling years. Though there was no significant correlation between virus prevalence and type of sampling site environment, OsHV-1 was more often detected in individuals collected from harbors perhaps due to not well identified stressors present in those sites. Furthermore, since the prevalence estimates are based on single time point samplings, no other environmental factors, such as temperature variations which can affect virus prevalence, were investigated.

Virus variability was investigated by analyzing three regions, ORFs 4/5, ORFs 35/38, and ORFs 42/43. The genomic diversity in double-stranded DNA viruses like herpesviruses is quite low due to the proofreading capability of DNA polymerases. However, we found a certain diversity within the OsHV-1 genome, with nine genotypes identified in 29 infected oysters and the omnipresence of a genotype very close to the µVar that appeared the most widespread type. Several mutations are expected to cause changes in amino acid sequences and to modify virus phenotypes. Though less than in the C region, ORFs 42/43 showed interesting variations, as demonstrated in vertebrate herpesvirus IAP genes (Earnshaw et al., 1999). In contrast, a study based on the analysis of the IAP genes on five specimens from Normandy and one from Ireland found a low discrimination power of these regions (Martenot et al., 2013). The ORFs 35/36/37/38 region was characterized by a large indel in a coding area in most specimens. The interpretation of this deletion from a phylogenetic perspective is quite problematic as with the usual models and algorithms, if we decide to exclude the automatic gap-filling option that considers the deletion as missing data, the indel would be treated as multiple evolutionary events when it actually more probably corresponds to a single event. In the present case, however, this deletion represents the complete loss of two genes and the partial loss of a third one, generating a remarkable phenotypical change. Given these circumstances, we included the indel as it improved analysis resolution. The use of sophisticated algorithms for alignment and phylogenetical analysis, adapted to take into account DNA-virus evolution, would be necessary in order to obtain accurate results with the correct phylogenetic distances (Nagy et al., 2012; Redelings and Suchard, 2007). Interestingly, the amplification of this region in the specimens from Capoiale never succeeded, suggesting the presence of a mutation, whereas the other markers belonged to the µVar cluster.

As reported in other studies, the C region was the most variable area with evidence of seven variants. Nevertheless, the number of trinucleotide repetitions was found to be insufficient to discriminate cluster membership, as the microsatellite areas are known for their excessively high variability. Thanks to the availability of a high number of sequences of the C region from around the world, we observed that the genotypes closely related to the µVar are all from Europe, except two South Korean specimens isolated in larvae, in a hatchery. The OsHV-1 reference, OsHV-1-SB and the AVNV are classified in the same group, phylogenetically distant from the µVar cluster and bearing a majority of specimens isolated from non *Ostreidae* bivalves. Two Italian samples, close to these genotypes, seemed to bear a large indel upstream of the microsatellite area, resembling the Var genotype (Arzul, 2001). These two genotypes resulted however in individual branches. Considering the specimens with the three markers available, these two well-separated clusters were also revealed in the two other targeted regions, and concatemers, highlighting a high divergence between the two virus groups. The simultaneous presence of ORFs 35/36/37/38 amplicons of two different lengths in the same sample (two cases) suggests polyinfections with different OsHV-1 genotypes. Recombination is another virus capability that we cannot exclude for OsHV-1. For these reasons, careful use of concatemers is warranted. Moreover, assembling complete genomes in the presence of many indels and polyinfections may be very difficult. The impact of these mutation events may be variously interpreted as the acquisition of virulence factors (insertions) or improvements in replication speed (loss of genome portions). The present study highlights future areas of focus: determination of the pathogenicity of the different genotypes, their target host species, and the demonstration, mainly at low viral load, of the presence of the virus within oyster tissues/cells. Furthermore, the weighty differences between the two clusters underline the need for a better description of the µVar genotype by complete genome sequencing.

The genetic selection of resistant oyster strains, imported from the native area of *C. gigas*, has been suggested as a solution against the problem of mortality events. The finding of healthy individuals, infected by OsHV-1 µVar-related genotypes, could indicate that resistant populations are also present in natural environment in Europe avoiding the importation of East Asian individuals and the risk of introduction of hitchhiking species and pathogens. However, in this regard, it is also acceptable that, in natural environment, individuals do not suffer the crowded and stressful rearing conditions that increase their susceptibility to the virus and facilitate its transmission.

5. Conclusions

Detection of infection with OsHV-1 in wild stocks of Pacific oyster *C. gigas* collected from sampling sites along the Adriatic, also in areas far from oyster farming or experimental trial sites, suggests a relatively remote introduction of the pathogen. Nine genotypes were characterized and a geographical distribution of some variants was observed. The present herpesviruses were allocated in two different clusters, showing high divergence. Two Italian genotypes, included in the cluster OsHV-1 reference/OsHV-1-SB/AVNV, showed a substantial distance with respect of the other European and East Asian genotypes. The others formed a well-defined group, comprising all the closely related European microvariants. This study improves the description of OsHV-1 epidemiology and demonstrates a high diversity of this virus in natural environments in Italy, a circumstance that has been reported to date only in East Asia (Mineur et al., 2015). The low diversity described in the majority of European studies, also in individuals outside of mortality events (Martenot et al., 2015), may be due to the isolation of viruses from farmed oysters where culture conditions reduce the genetic diversity of hosts and viruses alike. In the light of the present results, it appears necessary to intensify the survey efforts on European wild oyster stocks and to extend the study to more bivalve species to infer the herpesviruses origin.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2016.05.004.

References

Arzul, I., 2001. Herpès virus infectant les bivalves marins: détection, génome et transmission. Thèse.
Arzul, I., Nicolas, J.L., Davison, A.J., Renault, T., 2001. French scallops: a new host for Ostreid Herpesvirus-1. Virology 290 (2), 342–349.
Bai, C., Wang, C., Xia, J., Sun, H., Zhang, S., Huang, J., 2015. Emerging and endemic types of Ostreid herpesvirus 1 were detected in bivalves in China. J. Invertebr. Pathol. 124, 98–106.
Bucklin, A., Steinke, D., Blanco-Bercial, L., 2011. DNA barcoding of marine Metazoa. Annu. Rev. Mar. Sci. 3, 471–508.

Cochennec-Laureau, N., Baud, J.P., Bedier, E., Boudry, P., Huvet, A., Nicolas, J.L., Pépin, J.F., Petton, B., 2009. "Journées Surmortalités des huitres creuses, *Crassostrea gigas*" du programme P7 "Aquaculture Durable" des 8–9 décembre 2009 <http://sd2.archivehost.com/membres/up/214153133033562310/IFREMER_8_et_9_decembre_2009.pd>.

Comps, M., Duthoit, J.L., 1976. Infection virale associée à la "maladie des branchies" de l'huître portugaise *Crassostrea angulata*. C.R. Hebdomadaire Séanc. l'Acad. Sci. 283, 1595–1596.

Davison, A.J., Trus, B.L., Cheng, N., Steven, A.C., Watson, M.S., Cunningham, C., Le Deuff, R.M., Renault, T., 2005. A novel class of herpesvirus with bivalve hosts. J. Gen. Virol. 86 (1), 41–53.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order *Herpesvirales*. Arch. Virol. 154 (19), 171–177.

Dundon, W.G., Arzul, I., Omnes, E., Robert, M., Magnabosco, C., Zambon, M., Gennari, L., Toffan, A., Terregino, C., Capua, I., Arcangeli, G., 2011. Detection of type 1 Ostreid Herpes variant (OsHV-1 µVar) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. Aquaculture 314 (1–4), 49–52.

Earnshaw, W.C., Martins, L.M., Kaufmann, S.H., 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68, 383–424.

European Commission (EC), 2010 Commission Regulation 175/2010 of 2 March 2010 <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:052:0001:0013:EN:pdf>.

European Food Safety Authority (EFSA), 2015. EFSA Panel on Animal Health and Welfare (AHAW) 5 May 2015 <http://www.efsa.europa.eu/en/efsajournal/pub/4122>.

Farley, C.A., Banfield, W.G., Kasnic Jr., G., Foster, W.S., 1972. Oyster herpes-type virus. Science 178, 759–760.

Food and Agriculture Organization of the United Nations (FAO), 2014. Fisheries and Aquaculture Department, Dataset Global Aquaculture Production. On-line Query.

Friedman, C.S., Estes, R.M., Stokes, N.A., Burges, C.A., Hargove, J.S., Barber, B.J., Elston, R.A., Burreson, E.M., Reece, K.S., 2005. Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. Dis. Aquat. Org. 63, 33–41.

Grijalva-Chon, J.M., Castro-Longoria, R., Ramos-Paredes, J., Enríquez-Espinoza, T.L., Mendoza-Cano, F., 2013. Detection of a new OsHV-1 DNA strain in the healthy Pacific oyster, *Crassostrea gigas* (Thunberg) from the Gulf of California. J. Fish Dis. 36, 965–968.

Hine, P.M., Wesney, B., Hay, B.E., 1992. Herpes virus associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*. Dis. Aquat. Org. 12, 135–142.

Hwang, J.Y., Park, J.J., Yu, H.J., Hur, Y.B., Arzul, I., Couraleau, Y., Park, M.A., 2013. Ostreid herpesvirus 1 in farmed Pacific oyster larvae *Crassostrea gigas* (Thunberg) in Korea. J. Fish Dis. 36 (11), 369–372.

International Ostreid Herpesvirus 1 µVar Workshop, 2011. Final Report. Cairns, Queensland, Australia, 9–10 July 2011.

Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S.A., Gu, X., Read, A., Go, J., Dove, M., O'Connor, W., Kirkland, P.D., Frances, J., 2013. Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis. Aquat. Organ. 105 (2), 109–126. http://dx.doi.org/10.3354/dao02623.

Le Deuff, R.M., Nicolas, J.L., Renault, T., Cochennec, N., 1994. Experimental transmission of a herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. Bull. Eur. Ass. Fish Pathol. 14 (2), 69–72.

Lynch, S.A., Carlsson, J., Reilly, A.O., Cotter, O., Culloty, S.C., 2012. A previously undescribed ostreid herpes virus 1 (OsHV-1) genotype detected in the Pacific oyster, *Crassostrea gigas*, in Ireland. Parasitology 139 (12), 1526–1532.

Manzoni, P., 2010. Grande enciclopedia illustrata dei Crostacei dei Molluschi e dei Ricci di mare. Eurofishmarket 3 dicembre 2010.

Martenot, C., Oden, E., Travaillé, E., Malas, J.P., Houssin, M., 2010. Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. J. Virol. Methods 170, 86–89.

Martenot, C., Oden, E., Travaillé, E., Malas, J.P., Houssin, M., 2011. Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. Virus Res. 160, 25–31.

Martenot, C., Fourour, S., Oden, E., Jouaux, A., Travaillé, E., Malas, J.P., Houssin, M., 2012. Detection of the OsHV-1 µVar in the Pacific oyster *Crassostrea gigas* before 2008 in France and description of two new microvariants of the Ostreid Herpesvirus (OsHV-1). Aquaculture 338–341, 293–296.

Martenot, C., Travaillé, E., Lethuillier, O., Lelong, C., Houssin, M., 2013. Genome exploration of six variants of the Ostreid Herpesvirus 1 and characterization of large deletion in OsHV-1 µVar specimens. Virus Res. 178, 462–470.

Martenot, C., Lethuillier, O., Fourour, S., Oden, E., Trancart, S., Travaillé, E., Houssin, M., 2015. Detection of undescribed ostreid herpesvirus 1 (OsHV-1) specimens from Pacific oyster, *Crassostrea gigas*. J. Invertebr. Pathol. 132, 182–189.

Mineur, F., Le Roux, A., Maggs, C.A., Verlaque, M., 2014. Positive feedback loop between introductions of non-native marine species and cultivation of oysters in Europe. Conserv. Biol. 28, 1667–1676.

Mineur, F., Provan, J., Arnott, G., 2015. Phylogeographical analyses of shellfish viruses: inferring a geographical origin for ostreid herpesviruses OsHV-1 (*Malacoherpesviridae*). Mar. Biol. 162, 181–192.

Minson, A.C., Davison, A., Eberle, R., Desrosiers, R.C., Fleckenstein, B., McGeoch, D.J., Pellet, P.E., Roizman, B., Studdert, M.J., 2000. Family Herpesviridae. In: Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses, Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 203–225.

Morrissey, T., McCleary, S., Collins, E., Henshilwood, K., Cheslett, D., 2015. An investigation of Ostreid Herpes Virus microvariants found in *Crassostrea gigas* oyster producing bays in Ireland. Aquaculture 442, 86–92.

MPI, 2010. Range of Factors at Play in Oyster Deaths. 8 December 2010. Biosecurity in New Zealand News. Internet Communication. Ministry of Primary Industries of New Zealand <http://www.biosecurity.govt.nz/media/08-12-2010/range-of-factors-at-play-in-oyster-deaths>.

Nagy, L.G., Kocsubé, S., Csanádi, Z., Kovács, G.M., Petkovits, T., Vágvölgyi, C., Papp, T., 2012. Re-Mind the gap! Insertion-deletion data reveal neglected phylogenetic potential of the nuclear ribosomal internal transcribed spacer (ITS) of fungi. PLoS ONE 7 (11), e49794.

Nicolas, J.L., Comps, M., Cochennec, N., 1992. Herpes-like virus infecting Pacific-oyster larvae, *Crassostrea gigas*. Bull. Eur. Ass. Fish Pathol. 12 (1), 11–13.

Oden, E., Martenot, C., Berthaux, M., Travaillé, E., Malas, J.P., Houssin, M., 2011. Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: determination of a viral load threshold to prevent summer mortalities. Aquaculture 317, 27–31.

Office International des Epizooties (OIE), 2013. Infection with Ostreid Herpesvirus 1 Microvariant. Aquatic Manual, May 2013.

Peeler, E.J., Reese, R.A., Cheslett, D.L., Geoghegan, F., Power, A., Thrush, M.A., 2009. Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 µVar in the Republic of Ireland in 2009. Prev. Vet. Med. 105 (1–2), 136–143.

Redelings, B.D., Suchard, M.A., 2007. Incorporating indel information into phylogeny estimation for rapidly emerging pathogens. BMC Evol. Biol. 7, 40.

Ren, W., Chen, C.H., Renault, T., Cai, Y., Bai, C., Wang, C., 2013. Complete genome sequence of Acute Viral Necrosis Virus associated with massive mortality outbreaks in the Chinese scallop *Chlamys farreri*. Virol. J. 10, 110.

Renault, T., Cochennec, N., Le Deuff, R.M., Chollet, B., 1994a. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. Bull. Eur. Ass. Fish Pathol. 14 (2), 64–66.

Renault, T., Le Deuff, R.M., Cochennec, N., Maffart, P., 1994b. Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France – comparative study. Rev. Med. Vet. 145 (10), 735–742.

Renault, T., Le Deuff, R.M., Cochennec, N., Chollet, B., Maffart, P., 1995. Herpes-like viruses associated with high mortality levels in larvae and spat of Pacific oysters, *Crassostrea gigas*: a comparative study, the thermal effects on virus detection in hatchery-reared larvae, reproduction of the disease in axenic larvae. Vet. Res. 26, 539–543.

Renault, T., Arzul, I., 2001. Herpes-like virus infections in hatchery-reared bivalve larvae in Europe: specific viral DNA detection by PCR. J. Fish Dis. 24, 161–167.

Renault, T., Allain, G., Arzul, I., Chollet, B., Cobret, L., De Decker, S., Faury, N., Ferrand, S., François, C., Gracia, C., Haffner, C., Joly, J.P., Michel, J., Miossec, L., Morga, B., Nicolas, J.L., Omnes, E., Pépin, J.F., Robert, M., Saulnier, D., Schikorski, D., Segarra, A., Tourbiez, D., 2009. Summer mortality outbreaks of French Pacific oyster, *Crassostrea gigas*. In: 2008: Research and Detection of Pathogens. Dis. Fish and Shellfish, 14th International Conference of the EAFP, September, 14–19, Prague, Czech Republic, pp. 112–127. Abstract O.

Renault, T., Moreau, P., Faury, N., Pépin, J.F., Segarra, A., Webb, S., 2012. Analysis of clinical Ostreid Herpesvirus 1 (Malacoherpesviridae) specimens by sequencing amplified fragments from three virus genome areas. J. Virol. 86 (10), 5942–5947.

REPAMO (REseau de PAthologie des MOllusques), 2010. Bilan 2010 du réseau REPAMO Rapport interne IFREMER <http://wwz.ifremer.fr/repamo/Documentation/Rapports-d-activites>.

Rosani, U., Varotto, L., Domeneghetti, S., Arcangeli, G., Pallavicini, A., Venier, P., 2014. Dual analysis of host and pathogen transcriptomes in ostreid herpesvirus 1-positive *Crassostrea gigas*. Environ. Microb. http://dx.doi.org/10.1111/14622920.12706.

Saitou, N., Nei, M., 1987. The neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection and description of a particular Ostreid Herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res. 153 (1), 92–99.

Shimahara, Y., Kurita, J., Kiryu, I., Nishioka, T., Yuasa, K., Kawana, M., Kamaishi, T., Oseko, N., 2012. Surveillance of type 1 Ostreid Herpesvirus (OsHV-1) variants in Japan. Fish Pathol. 47 (4), 129–136.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 10, 2731–2739.

Terol, J., Mascarell, R., Fernandez-Pedrosa, V., Pérez-Alonso, M., 2002. Statistical validation of the identification of tuna species: bootstrap analysis of mitochondrial DNA sequences. J. Agric. Food Chem. 50, 963–969.

Vasquez-Yeomans, R., Caceres-Martinez, J., Figueras-Huerta, A., 2004. Herpes-like virus associated with eroded gills of the Pacific oyster *Crassostrea gigas* in Mexico. J. Shellfish Res. 23, 417–419.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D.N., 2005. DNA barcoding Australia's fish species. Philos. Trans. R. Soc. B 360, 1847–1857.
Webb, S.C., Fidler, A., Renault, T., 2007. Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): application in a survey of New Zealand molluscs. Aquaculture 272 (1–4), 126–139.
Xia, J., Bai, C., Wang, C., Song, X., Huang, J., 2015. Complete genome sequence of Ostreid herpesvirus-1 associated with mortalities of *Scapharca broughtonii* broodstocks. Virol. J. 12, 110.

2.1.3. Is OsHV-1 detectable in other mollusc species in recognised infected areas?

The research of OsHV-1 in other mollusc species, naturally present in coastal marine environments, has a double purpose. Firstly, we wanted to determine if these wild populations could represent a risk of viral infection for OsHV-1 microvariants-free oyster spat placed in farming areas, acting as reservoir species. The second aim was to evidence if these species are affected by specific variants of OsHV-1.

The DNA extraction procedure is known to be challenging in molluscs due to their high amount of secreted mucopolysaccharides and polyphenolic proteins that copurify with DNA (Winnepenninnckx B. *et al.*, 1993). In the present study we applied the extraction method performed for oyster DNA in the previous chapters in which we used the QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol for blood or body fluids. The efficiency of the extraction has been evaluated.

Materials and Methods

To verify these hypotheses, the presence of OsHV-1 was investigated in 678 individuals of 12 different mollusc species (Table 3), collected in the same areas where *C. gigas* wild populations resulted to be infected by OsHV-1, as highlighted in Publication 1. Two other sites were included: San Teodoro and La Spezia, located in the Tyrrhenian Sea. No wild populations of Pacific oysters were recorded in these two sites during our sampling campaigns. However, Pacific oyster farming has been carried out in these locations for numerous years and infection by OsHV-1 has been evidenced several times in reared individuals, suggesting that the pathogen is already present in the environment. Eleven of the twelve collected species are bivalves: *Aequipecten opercularis* (N=2), *Anomia ephippium* (N=50), *Chlamys glabra* (N=8), *Chlamys varia* (N=20), *Limaria tuberculata* (N=53), *Mytilus* sp. (N=270), *Ostrea edulis* (N=91), *Parvicardium* sp. (N=11), *Ruditapes decussatus* (N=50), *Ruditapes philippinarum* (N=100), and *Solen marginatus* (N=6). We presume that mussels of the genus *Mytilus* were *M. galloprovincialis* but the distinction on the basis of morphological features is not always discriminant. Gastropods have also been collected and identified as *Patella* sp. (N=29).

Soft tissues of each individual have been minced using the same protocol applied to oysters, but, in this case, part of the digestive gland was added to the pool as no preliminary study has been conducted to define the best target organ for the detection of OsHV-1.

Extraction of 50±0.5 mg of minced tissues was carried out as reported in Appendix B.1. Since we did not possess information on the efficiency of this extraction protocol in other mollusc species than *C. gigas*, purity and concentration of the extracted DNA was checked with a NanoDrop® 2000c spectrophotometer (ThermoFisher Scientific®, Waltham, MA, USA) by reading the whole absorption spectrum (220–320 nm) and absorbance ratio at 260/280 and 230/260 nm (Wilfinger *et al*., 1997).

OsHV-1 detection was carried out using a real-time PCR protocol based on TaqMan® technology (Martenot *et al*., 2010) as illustrated in Appendix B.2. This protocol includes the use of an Internal Control IC to exclude false negative results imputable to the presence of PCR inhibitors. A negative control was included and consisted of 2 µL of DNA/nuclease-free water in 23 µL of real-time PCR mix. The extraction control was treated exactly as a sample. The standard curve was obtained using 2 µL of different dilutions from 10 to 10^5 copies of OsHV-1 DNA units/µL of plasmidic DNA solution, corresponding to the OsHV-1 targeted region, and acting also as positive control. The run was considered valid only if the extraction and the negative controls did not present any amplification, and if the regression coefficient of the standard curve was at least 0.98 and the slope between -4.115 and -2.839. A sample was considered positive only if the Ct value was ≤38.5. A sample was considered a true negative only if amplification of the IC was successful.

Results

The quantity of DNA extracted was satisfying for all the mollusc species, except for *S. marginatus* and *Patella* sp. with a DNA concentration in the extract of 224.8 and 124.8 ng μl^{-1} respectively, lower if compared to other species. The values of the ratios 260/280 and 260/230 ranged from 1.80 to 2.00 and from 0.61 to 2.20 respectively. In fact, a low 260/230 ratio has been observed for all the specimens of *Patella* sp. and *S. marginatus*, and to a lesser extent *L. tuberculata*. The results of Nanodrop analysis are reported in Table 7. An uneven quantity of extracted DNA has been observed beetween the different mollusc species, ranging from an average of 224.75 ng/µL in *S. marginatus* to 1068.44 ng/µL in *Mytilus* sp. Surprisingly, none of the samples tested during the present study tested positive for OsHV-1. As a successfuly amplification of the IC was obtained for all of them, except for the two specimen of *S. marginatus* from Caleri, the presence of a PCR inhibitor was excluded.

Species	Mean DNA concentration ng µL^{-1}	Concentration range ng µL-1	Range 260/280	Range 260/230
Aequipecten opercularis	520.9	488.2-564.5	1.86-1.88	1.78-1.91
Anomia ephippium	643.0	561.6-732.5	1.90-1.98	1.64-1.86
Chlamys glabra	557.8	492.9-638.0	1.96-2.00	1.74-1.87
Chlamys varia	589.6	493.2-625.4	1.92-1.99	1.65-1.86
Limaria tuberculata	509.3	475.1-539.9	1.91-1.95	1.56-1.75
Mytilus sp.	1068.4	1013.2-1167.1	1.89-1.97	1.91-2.20
Ostrea edulis	582.8	529.0-653.2	1.87-1.93	1.91-2.13
Parvicardium sp.	801.5	784.6-832.5	1.78-1.94	1.87-2.04
Ruditapes decussatus	783.7	728.4-861.4	1.80-1.90	1.90-2.12
Ruditapes philippinarum	813.7	724.5-881.8	1.83-1.92	1.80-2.05
Solen marginatus	224.8	202.3-245.8	1.91-1.93	1.21-1.23
Patella sp.	158.8	148.7-181.3	1.84-1.94	0.61-0.74

Table 7 Concentration and purity of DNA extracted from 50 mg of minced tissues from various mollusc species.

Discussion and conclusions

The use of the extraction kit QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands) with the protocol for blood or body fluids gave generally good results, with expected DNA concentration and purity. The differences observed between the various species in the amount of the DNA extracted may be easily explained by their uneven genome length, the different architecture of their tissues that influences the number of cells, and quantity of energy reserves within the tissues. However, a poor efficiency of this method was evidenced when applied on the gastropod *Patella* sp. and on the *Solenidae* bivalve *S. marginatus*. In these two species both DNA concentration and purity resulted insufficient. The ratio 260/230, in particular, showed very low values suggesting that contaminants are present, potentially phenols and chaotropic salts that are able to denature DNA and enzymes such as the DNA polymerase used for the PCR. In fact, the amplification of the IC resulted unsuccessful for the samples of *S. marginatus.*

On the contrary, in the samples from other species, the results of the real-time PCR for the detection of OsHV-1 were validated by the obtainment of the IC amplification. Interestingly, all individuals tested negative for OsHV-1. We showed previously that the virus was present in *C. gigas* in these sampling areas and, moreover, as we will illustrate in the chapter 2.3, farmed populations of *C. gigas* in Caleri and San Teodoro experimented high viral loads in the same period than wild mollusc sampling campaigns.

Nevertheless, even if the real-time PCR method has high sensitivity (detection limit 6 GU/mg of tissues) for detecting OsHV-1 (Martenot *et al.,* 2010), we are not able to

demonstrate that it is sufficient to evidence the positivity in the putative latent phase. Moreover, the seasonality of the physiological cycle and consequently the susceptibility to diseases varies among the different mollusc species. This could also induce a different seasonality of the activation/reactivation of the herpesvirus, leading to the establishment of viral cycle based on the successive infection of different mollusc species during the year improving its survival. To exclude this unlikely hypothesis, a year-round study could be considered.

Another important topic to develop in future studies is the problem of nucleic acid extraction in most mollusc species. Development of adequate methods for DNA extraction is of primary interest because it represents the first step of all the genetic analyses.

2.2. Sequencing of the complete genome of OsHV-1 µVar

The present study intended to obtain the complete genome sequence of the Ostreid herpesvirus variant termed µVar. In fact, until now and despite the global economic impact of this genotype on Pacific oyster production, its full genome sequence was still unknown. The results have been submitted to the scientific journal Virology and the author's manuscript is reported at the end of this chapter.

Publication 2: **Complete genome sequence of Ostreid herpesvirus type 1 µVar isolated during mortality events in the Pacific oyster *Crassostrea gigas* in France and Ireland. Virology, under review.**

In 2005, Davison *et al*. published the complete genome sequence of the Ostreid herpesvirus type 1 (OsHV-1) obtained from infected larvae collected in France in 1995 using a cloning-based method. This sequence was considered as the reference sequence and the strain termed OsHV-1 "reference". Since then, two other whole genome sequences of closely-related strains have been issued, through a primer walking method: the AVNV (Ren *et al*., 2013) and OsHV-1-SB, associated with mortalities in *Chlamys farreri* and *Scapharca broughtonii*, respectively. The "primer walking" is a sequencing method for long fragments, based on the use of primer pairs designed by referring to a sequence used as mould, in order to generate consecutive and slightly overlapping sequences. The obtainment of good results with this method requires high homologies between the reference and the analysed sequences.
Later, after the description of the new variant µVar associated with numerous mass mortality events in commercially produced Pacific oysters since its first detection in 2008, few studies have been conducted on some restricted genomic areas of the virus, highlighting some differences between the reference and the µVar genotypes (Martenot *et al*, 2013). However, despite the economic relevance of the OsHV-1 µVar and its evident diversity with OsHV-1 reference, its complete genome sequence was not available yet.
Considering that the obtainment of the full sequence of the viral genome represents an important step for numerous subsequent studies on OsHV-1 µVar virulence, phylogeny, and gene expression, we developed a workflow to achieve this aim.

The cloning-based method is an efficient and consolidated approach for full genome sequencing, but it is laborious and time-expensive. Moreover, since bivalve cell lines are not yet available for the propagation and isolation of OsHV-1, high amounts of exogenous DNA are present in the samples, even after purification protocols. Thus, in the present study, thanks to the development of “next-generation” sequencing (NGS) technologies and advances in diagnostic virology, we sequenced the µVar whole genome *via* a “sequencing by synthesis” (SBS) approach, by applying the Illumina reversible termination sequencing strategy. For the data analysis we performed a reference-assisted assembly. The reads were aligned on the OsHV-1 reference sequence, using Bowtie 2 version 2.2.9 (Langmead & Salzberg, 2012) and the non-aligned reads were analysed using the MindTheGap software (Rizk *et al.*, 2014), assembled, and the exact position of the resultant sequence was identified. In order to evaluate the opportunity of a preliminary purification, we also tested the effects of different sample purification methods on sequencing quality.

Our results demonstrated the suitability of the developed workflow for the sequencing of long-length genome of dsDNA viruses, also from unpurified samples, such as what observed with OsHV-1 µVar. The final genome length of OsHV-1 µVar was approximately 205 Kbp, shorter than the reference genotype and the overall genome organization resembled herpes simplex viruses. 94.4% similarity was observed with the OsHV-1 reference genotype. Large indels, including five deletions and three insertions were found to induce the loss and the addition of several ORFs, summed with codon substitutions in 64% of the genes shared with the reference type. The layout of ORFs in the OsHV-1 µVar genome is represented in Figure 28.

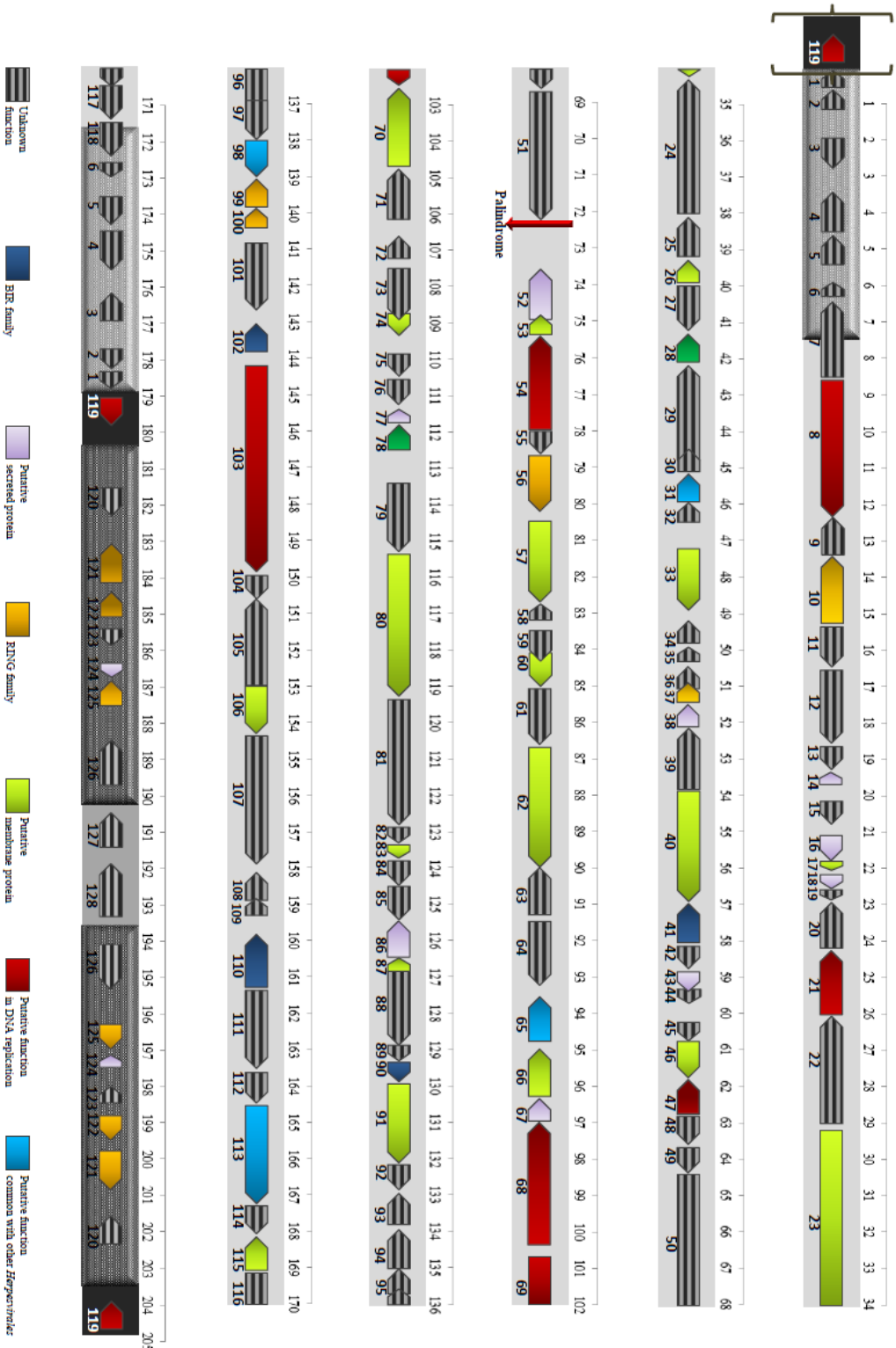

Fig. 28 Layout of Open Reading Frames in the OsHV-1 µVar genome. ORF numeration is assigned under each ORF. The direction of arrows, representing ORFs, indicates if the gene is located in the upper or lower strand. The scale above the genome is in kbp**.**

Complete genome sequence of Ostreid herpesvirus type 1 µVar isolated during mortality events in the Pacific oyster *Crassostrea gigas* in France and Ireland

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Abstract

Infections with Ostreid herpesvirus 1 (OsHV-1) microvariants in young Pacific oysters are associated with massive mortality events and significant economic losses. Previous studies, focusing on few regions of the genome, have revealed the genomic diversity of these genotypes with respect to the reference type. We used a NGS process to sequence the whole genome of the OsHV-1 µVar in infected individuals, collected during mortality events in France and Ireland. The final genome length of OsHV-1 µVar was approximately 205 Kbp, shorter than the reference genotype and the overall genome organization resembled herpes simplex viruses. 94.4% similarity was observed with the OsHV-1 reference genotype. Large indels, including five deletions and three insertions were found to induce the loss and the addition of several ORFs, summed with codon substitutions in 64% of genes shared with the reference type. This diversity raises the question of the exact origin and evolution of OsHV-1 µVar.

Keywords Ostreid herpesvirus-1 µVar, complete genome, indels, NGS, *Crassostrea gigas*

Introduction

The worldwide production of animal species in aquaculture is estimated at 74 million tons (FAO, 2014), of which molluscs account for 22% of total production and cupped oysters *Crassostrea* spp. alone make up more than 30% of mollusc aquaculture. In recent years, mass mortality events involving essentially young specimens of Pacific oyster *Crassostrea gigas* have been associated with infection with Ostreid herpesvirus type 1 (OsHV-1) microvariants (EFSA, 2015). Infection with herpes-type viruses was first reported by Farley *et al*. in 1972 in *C. virginica* and since the early 1990s, the presence of herpes-like viruses was often associated

with mortality events in larval and juvenile stages of different mollusc species and, in the Pacific oyster *C. gigas* (Thunberg, 1793) in particular (Hine, 1992; Nicolas *et al*., 1992; Renault *et al*., 1994a, b; Vasquez-Yeomans *et al.*, 2004; Friedman *et al*., 2005; Burge *et al.*, 2006). The virus was classified as a member of the order *Herpesvirales* (Davison *et al*., 2009) and the species was named *Ostreid herpesvirus* (Minson *et al*., 2000). OsHV-1 is a member of the *Malacoherpesviridae* family and the single species of the genus *Ostreavirus* (Davison *et al*., 2009; ICTV, 2015). Three years after the complete genome sequencing of OsHV-1 (GenBank AY509253), the first to be described and considered as the reference genotype (Davison *et al*., 2005), a new viral variant, termed µVar, was identified by Segarra *et al*. (2010) as being responsible for the dramatic rise in mortality since April-May 2008 (Cochennec-Laureau *et al*., 2009; Renault *et al*., 2009), with mortality rates ranging between 60% and 100%. After a preliminary study targeting three regions of the genome, two showed polymorphisms and the µVar genotype was formally characterized by the deletion of 12 consecutive bp in a microsatellite locus upstream of ORF 4 (GenBank HQ842510) and by substitutions and indels upstream and in ORF 4, and ORFs 42/43. In most cases, the complete loss of ORFs 36 and 37 and the partial loss of ORF 38 were also observed (Segarra *et al*., 2010; Renault *et al*., 2012). Since then, the µVar and several other closely-related genotypes, subsumed under the term “microvariants” (OIE, 2014), have been described in *C. gigas* around the world, including Australia (Jenkins *et al*., 2013; Paul-Pont *et al*., 2014), China (Renault *et al*., 2012), France (Martenot *et al*., 2011, 2012; Renault *et al*., 2012), Ireland (Lynch *et al*., 2012; Peeler *et al*., 2012; Morrissey *et al*., 2015), Italy (Dundon *et al*., 2013; Burioli *et al*., 2016), Japan (Shimahara *et al*., 2012), Mexico (Grijalva-Chon *et al*., 2013), the Netherlands (Gittenberger *et al.*, 2016), New Zealand (Keeling *et al*., 2014), Norway and Sweden (Mortensen *et al.*, 2016), Portugal (Batista *et al*., 2015), South Korea (Hwang *et al.*, 2013) and Spain (Roque *et al*., 2012). An OsHV-1 microvariant was also associated with mortality events in adult individuals of *C. angulata* in Portugal (Batista *et al*., 2015).

The whole genome of four different virus specimens, belonging to the *Malacoherpesviridae* family has been sequenced. Three are members of the *Ostreavirus* genus: the reference OsHV-1 (Davison *et al.*, 2005) with a total length of 207,439 bp (GenBank AY509253), Acute Viral Necrosis Virus (AVNV), consisting of 210,993 bp (GenBank GQ153938) (Ren *et al*., 2013), considered a variant of OsHV-1 and associated with mortality outbreaks in *Chlamys farreri* in China, and another OsHV-1 variant affecting *Scapharca broughtonii* broodstocks named OsHV-1-SB (Xia *et al.*, 2015), 199,354 bp (GenBank KP412538); the fourth is an *Aurivirus*, the Abalone herpesvirus (AbHV-1) that infects abalone species (*Haliotis laevigata, H. rubra* and *H. diversicolor*) (Savin *et al*., 2010). OsHV-1 reference genome sequencing was carried out *via* a cloning method. The genomic organization is $TR_L/U_L/IR_L/X/IR_S/U_S/TR_S$ (Davison *et al*., 2005), where U_L (167,843 bp) and U_S (3,370 bp) are two unique regions flanked by repeat sequences in opposing orientation TR_L/IR_L (7,584 bp) and TR_S/IR_S (9,774 bp) and separated by a third unique X region (1,510 bp), resembling the herpes simplex viruses (HSV) (Kieff *et al*., 1971; Wagner & Summers, 1978) and Human cytomegalovirus (HCMV) (Weststrate *et al*., 1980; Davison *et al*., 2003). Both AVNV and OsHV-1-SB were sequenced with a PCR-based approach, the primer-walking technique, using the OsHV-1 reference sequence as template. Despite the relevance of the OsHV-1 µVar genotype, large epidemiological studies to date have focused only on a few regions of the viral genome (Renault *et al.*, 2012; Mineur *et al*., 2015), and its overall genome sequence remained unknown. With the development of “next-generation” sequencing (NGS) technologies and advances in diagnostic virology (Barzon *et al.*, 2011), it is now possible to obtain an enormous volume of sequences in a single run, at a reasonable cost and with a remarkable gain in time (Goodwin *et al*., 2016).

The purpose of the present work was to describe the novel genomic features of OsHV-1 µVar as compared to the reference genotype, with a view to better understand the reasons for its increased virulence. We sequenced the µVar whole genome *via* a sequencing by synthesis

(SBS) approach, by applying the Illumina reversible termination sequencing strategy (Li *et al*., 2003; Bentley *et al*., 2008). Since bivalve cell lines are not yet available for the propagation and isolation of OsHV-1 (Yoshino *et al*., 2013), we carried out analyses on samples from the tissues of infected young *C. gigas* collected during mortality events. Previous studies have demonstrated the suitability of the high throughput feature of NGS for pathogen genome sequencing in noncultured samples (Loman *et al*., 2013; Gire *et al.*, 2014). Since the major challenge in these conditions is the noise from host and microbiota DNAs, pretreatments are usually employed before sequencing. We also evaluated the effects of sample purification methods on sequencing quality.

Materials and Methods

Sample preparation and nucleic acid extraction

Sequencing analyses were performed on pools of tissues from four batches of oyster *C. gigas* spat, affected by mortalities induced by OsHV-1 µVar in France and Ireland, and stocked at -80°C. The batches were selected on the basis of their viral load (up to 10^7 genome units (GU)/50 mg of minced oyster soft tissue), origin and ploidy (Table 1). The viral load was initially determined using the method described by Martenot *et al.* (2010). Different nucleocapsid purification protocols, as suggested in previous studies (Davison & Davison, 1995; Le Deuff & Renault, 1999; Tan *et al.*, 2008; Dai *et al*., 2013; Dai & Zhou, 2014), were applied on tissues of the *Meuv* batch to enhance the OsHV-1 DNA to exogenous DNA ratio and then compared. The *Meuv* batch was chosen because of the higher quantity of stored tissues. Samples of 10 g of spat tissues were disrupted with an Ultra-Turrax® (Staufen, Germany) homogenizer was achieved under four different conditions (Table 2), all supplemented with phenylmethylsulfonyl fluoride 1mM (PMSF), a protease inhibitor. The four suspensions were clarified by centrifugation at 250 x g and then at 1000 x g and 4000 x g, at 4°C, for 30 min each. The supernatants were filtrated at 5 µm, 0.45 µm, and 0.22 µm. One half of the volume of each suspension was ultracentrifuged for 1 h at ~ 80,000 x g (SW

28 rotor, Beckman Coulter, Inc.) at 4°C, and the resulting pellet resuspended in 100 µL of phosphate buffered saline (PBS) followed by DNAse treatment (TURBO DNA-free™ Kit, ThermoFisher Scientific, Inc.). After a similar centrifugation step, the other half was resuspended in 12 mL of sterile artificial seawater supplemented with PMSF and 6 mL volumes of virus suspensions were gently loaded on series of discontinuous sucrose gradients 60% (6 mL), 50% (5 mL), 40% (6 mL), 30% (6 mL), and 10% (5 mL) (w/v) prepared in seawater, and ultraspun at ~80,000 x g at 4°C for 1 h. For each suspension, a 2 mL fraction from the two gradient tubes was collected at the 40-50% interface (Le Deuff & Renault, 1999), pooled, diluted fourfold with seawater and pelleted at 80,000 x g for 90 min at 4°C. Finally, the pellet was resuspended in 20 µL of PBS. Extraction was performed on the 100 µL purified suspensions treated with DNase (*MeuvPur1*), and on the 20 µL suspensions purified by gradient (*MeuvPur2*). In addition, considering the low viral DNA concentration in the purified suspensions that may prevent the achievement of optimum sequencing results, extraction was also conducted directly on 50 mg of minced spat tissues for all batches. Extraction was carried out using a QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol for blood or body fluids, except for elution performed in 60 µL Qiagen elution buffer (Martenot *et al*., 2010). The final OSHV-1 DNA concentration in the elutes was estimated using a qPCR assay based on TaqMan® technology (Applied Biosystems) (Martenot *et al*., 2010).

Qualification and quantification of DNA samples by NanoDrop and Qubit

Optimal quality of the extracted DNA was checked with a NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific™, Waltham, MA, USA) by reading the whole absorption spectrum (220–320 nm) and absorbance ratio at 260/280 and 260/230 nm (Wilfinger *et al*., 1997). The total concentration of double strand DNA was then quantified, for each sample, with the Qubit™ 2.0 Fluorometer (ThermoFisher Scientific™). The instrument was calibrated with a Quant-iT dsDNA HS Assay (declared assay range 0.2–100

ng; sample starting concentration between 10 pg/µL and 100 ng/µL) and a Quant-iT dsDNA BR Assay (declared assay range 2–1000 ng; sample starting concentration between 100 pg/µL and µg/µL), according to the manufacturer's instructions. The efficiency of the purification protocol was assessed roughly through approximation of the OsHV-1 DNA to exogenous DNA ratio calculated on the basis of the qPCR and Qubit™ results, and viral genome molar weight, considering a virus length of 207,400 bp with 38.7 % G-C content.

Library preparation and sequencing

Library preparation and sequencing were carried out at the Laboratory of Biology and Genetics of Cancer working team from the Centre François BACLESSE (Caen-France). DNA shearing was performed using an S220 Focused-ultrasonicator (Covaris™, Woburn, MA, USA) with settings adapted from those recommended by Illumina® (duty factor 10%, intensity 5.0, Cycles per burst 150, duration 45 s). The average fragment length was about 260 bp (Agilent 220 TapeStation, Santa Clara, CA, USA). The paired-end fragment library was prepared with a SPRIworks HT fragment library kit (Beckman Coulter, Inc.), according to the manufacturer’s instructions. A pre-capture PCR assay for library enrichment was performed under the following thermal cycling conditions: activation/initial denaturation 98°C for 3 min; 3 amplification cycles at 98°C for 10 s, 65°C for 1 min, 72°C for 1 min, and a final extension cycle at 72°C for 5 min, followed by purification with AMPure XP beads (Agencourt, Beckman Coulter, Inc.). DNA quantity and quality controls were validated using an Agilent 220 TapeStation and a Qubit™ 2.0 fluorometer. Sequencing was carried out on an Illumina® NextSeq® 500 system with a 2 x 150 bp Mid Output kit.

Data analysis

BCL conversion and demultiplexing were performed with bcl2fastq (Illumina®) software and read quality was checked with FastQC Version 0.11.4 (Babraham Bioinformatics). Read alignments to the OsHV-1 reference genome (GenBank AY509253) were done using Bowtie 2 version 2.2.9 (Langmead & Salzberg, 2012) (http://bowtie-

bio.sourceforge.net/bowtie2/index.shtmland). Alignment quality evaluation, post-processing conversions and generation of consensus sequences were carried out with Samtools (http://www.htslib.org/). Interactive Genome Viewer (IGV, Broad Institute) (Robinson *et al*., 2011; Thorvaldsdóttir *et al*., 2013) was used to determine the coverage at each locus, nucleotide variations and indels. MindTheGap (Rizk *et al.*, 2014) software was used to perform detection and assembly of insertions with respect to the reference genome. In order to ascertain the presence of these indels, *ad hoc* primers were designed with the aid of the Eurofins Genomics design tool (Ebersberg, Germany). Sequences are given in Table 3. Conventional PCR mixtures consisted of 25 µL of 2X QIAGEN® Multiplex Pre Mix, 1 µL of each primer (20µM), 19 µL of DNA/nuclease-free water, and 4 µL of extracted DNA eluate. The thermal program was as follows: activation/initial denaturation at 95°C for 15 min, 40 amplification cycles at 95°C for 30 s, at 57°C for 90 s, at 72°C for 90 s, and a final extension at 72°C for 3 min. Amplicons were sent to Eurofins MWG Operons (Ebersberg, Germany) for sequencing both the sense and the antisense strands. Prediction of Open reading frames (ORF) using NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was restricted to those larger than 100 and according to the criteria described by Davison *et al.* (2005). In addition to reading frame analysis, we verified the locations of potential transcriptional polyadenylation signals (AATAAA and ATTAAA) following each ORF. ORF similarities between µVar and reference genotype were calculated with EMBOSS Needle pairwise sequence alignment (EMBL-EBI, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK). The amino acid sequences were compared and analyzed using NCBI BLASTP (https://www.ncbi.nlm.nih.gov/) and InterPro (https://www.ebi.ac.uk/interpro/) (Mitchell *et al*., 2015). Finally, putative glycosylation site prediction and protein topology were carried out with Protter (http://wlab.ethz.ch/protter/#) (Omasits *et al*., 2014).

Results

Sample preparation, processing quality evaluation, alignment

The best OsHV-1 DNA to exogenous DNA ratio was obtained with the B' protocol, based on ultracentrifugation on sucrose gradient; however the total amount of residual viral DNA was very low, with less than 10 GU/µL, as observed in all the ultracentrifugation protocols that we applied, and a residual presence of exogenous DNA was confirmed. The protocols consisting in DNase treatment after 0.22 µm filtration gave less satisfying results with a dramatic decrease in total DNA concentration but a poor improvement in the target DNA ratio. Thus, given these results, the extracts from the purification protocols B and B' were chosen to be submitted to the sequencing workflow, in addition to the four DNA extracts from the unpurified samples. Library preparation of the six samples yielded an average fragment size of 264 bp. Sequencing with Illumina NextSeq yielded a total of 40 Gbases. The quality of the base calling, as determined by a quality score Q, was acceptable, with 100% of read1 and read2 sequences ≥Q30 (99.9% accuracy of base calling at a particular sequence position). No sequences were flagged as poor quality. Multiple alignment analyses with the reference virus sequence were conducted on the six samples. The main depth of coverage (DOC) ranged from 1400, for the *Meuv* and the *Dung* samples, to 10 for the *MeuvPur1* and *MeuvPur2* samples. Within the single sequence alignments, the DOC ranged in some regions from 0, suggesting deletions, to two times the main coverage (2900), implying that the region in question, unique in the reference genome, was present twice in the aligned genome. Finally, MindTheGap analysis gave usable results only with reads from the purified samples B and B', suggesting the presence of different insertions.

Genomic analysis of OsHV-1 µVar

All the sequenced specimens resulted µVar genotypes and presented the same set of mutations as reported by Segarra *et al.* (2010). In addition to the known deletion of four CTA in the C region, five large deletions were observed in the µVar genome as compared to the reference OsHV-1 genome (Davison *et al.*, 2005). On the basis of the nucleotide numeration of

sequence AY509253, they involved regions 17,707 to 19,092 (1386 bp), 52,253 to 52,858 (606 bp), 67,973 to 68,572 (600 bp), 93,120 to 96,669 (3550 bp), and 175,018 to 175,743 (726 bp). These deletions were also reported by Martenot *et al*. (2013) in a study on several ORFs of µVar. All are located in the U_L region, except the last straddling U_L and IR_L. Moreover, MindTheGap highlighted three insertions: an 86 bp insertion in the inverted repeat TR_L/IR_L, which was present twice (nucleotide 1655 and 181,357 of AY509253), and a third insertion of 2671 bp located in the U_L (base 60,740 of AY509253). We found two variants of OsHV-1 µVar, termed A and B, in our samples, that differed for 68 nucleotides but both carrying these large indels that were also detected by Sanger sequencing. Genome length was 204,886 bp and 204,897 bp for variant A (GenBank accession number KY242785) and variant B (GenBank accession number KY271630), respectively. Two pools were found to be infected with only one variant: variant A was detected in the pool from *Meuv* and variant B in the pool from *Dung*. Two other pools, *Géfo* and *Blai*, were infected with both variants. However, variant A appeared to be the more common one, accounting for about 75% of the genotypes in these samples. The main coverage value was 1400 (in *Meuv* and the *Dung* samples), except for the X region that showed twice the coverage value in all samples, suggesting the presence of two copies of X along the genome in the majority of specimens. This observation, contrasts with Davison *et al*. (2005) who found only a small proportion of molecules containing two copies of X. Since the exact position of X' was not determined, the genome organization may be represented as TR_L-U_L-IR_L-X-IR_S-U_S-TR_S-X' or X'-TR_L-U_L-IR_L-X-IR_S-U_S-TR_S. Nevertheless, lower quality and coverage in read alignment were observed in the junctions between these different regions as compared to the rest of the genome. The lower coverage at the junctions may be explained by the usual presence of four isomers that differ in the relative orientation of the unique regions, as observed in other herpesviruses such as HSV-1 and HCMV (Hayward *et al.*, 1975; Weststrate *et al*., 1980). In fact, Davison *et al*. (2005) reported that OsHV-1 genomes exist as four isomers, present in

approximately equimolar amounts in samples. Of the two unique regions, U_S was confirmed to be 3370 bp and U_L resulted shorter than the reference genotype (164,268 bp). The inverted repeat TR_S/IR_S, flanking the short unique region U_S, and TR_L/IR_L, framing U_L, were 9776 bp and 7338 bp in length respectively, in variant A, whereas in variant B, TR_L/IR_L were slightly longer, with five nucleotides more in non-coding regions. The X region was 1510 bp long, like the reference genotype. The deletion of the large palindrome in the U_L region observed by Ren *et al*. (2013) in AVNV was not confirmed in the present study. This figure, as compared to the Ori_L sequence of vertebrate herpesviruses such as *Alphaherpesvirinae* and HSV-1 in particular (Weller *et al*., 1985), is a presumptive origin of DNA replication. The OsHV-1 µVar has a nucleotide composition of 38.9% G+C, comparable to the reference type (38.7%). The nucleotide sequence of the OsHV-1 µVar genome showed 94.4% similarity with the reference OsHV-1 reference (AY509253), 94.0% with AVNV (GQ153839), and 89.3% with OsHV-1 SB (KP412538).

Coding regions

ORFs of the OsHV-1 µVar variant A (ORF^V) were compared to the reference genotype ORFs (ORF^R) (Table 4). The indels described above led to the complete loss of five ORFs with respect to OsHV-1 reference type (ORF^Rs 36, 37, 48, 62, and 63), while three ORFs resulted shortened (ORF^Vs 12, 34, and 118), and ORF^V 36 was longer. The 2671 bp insertion led to the addition of four ORFs (ORF^Vs 43 to 46) in the U_L. The disrupted genes (ORF^Rs 5, 32, 50, 65, 73, and 105), as described by Davison *et al*. (2005), were considered as the different resultant putative ORFs. As suggested by Davison *et al.* (2005), we added ORF^V 17 to the ORF set, though shorter than 100 codons, because it encodes a putative membrane protein. ORF^Vs 43 and 44, located in the 2671 bp insertion, were both retained because they overlap for only three nucleotides. Our analysis suggests the presence of 128 ORFs, 13 of which are duplicated because situated in the inverted repeats (Figure 1). ORF length varied between 76 and 1878 codons. The main similarity, between the reference genotype and the µVar ORFs,

was 98%, ranging from 57.6% to 100% (Table 4). 35 % of the 113 µVar ORFs, with a correspondent gene in the reference genotype, were identical to the reference ORF.

Different genes, some of which encoding putative functions common to other herpesviruses, were predicted (Table 4). Several genes, involved in DNA replication, as observed in HSV-1, were probably identified, such as for ORF^{V}s 21 and 54, that encode the small and large subunits of Ribonucleotide Reductase (Cohen, 1972), ORF^{V}s 8, 68, and 69 that encode the Helicase/Primase Complex (Dutia, 1983; Crute *et al.*, 1989), ORF^{V} 47 that bear a Deoxynucleoside Kinase domain (Dubbs *et al.*, 1964), ORF^{V} 103 that encode the catalytic subunit of the replicative DNA Polymerase (Chartrand *et al*., 1980), ORF^{V} 119 that encode the Replication Origin-binding Protein (Elias *et al*., 1986). Functional information, compatible with herpesvirus characteristics, were also provided for ORF^{V} 113 that encode the Large subunit DNA-Packing Terminase (Davison, 2002), ORF^{V} 65 that encode an RNA-Ligase (Silber *et al*., 1972), and ORF^{V} 98 that is thought to produce an Exonuclease. ORF^{V} 31 was found to be related to the UL92 in HCMV (subfamily *Betaherpesvirinae*) and to ORF 31 in Kaposi Sarcoma-associated herpesvirus (subfamily *Gammaherpesvirinae*). Four ORFs contained Baculovirus Inhibitor of apoptosis (IAP) Repeat (BIR) domains (ORF^{V}s 41, 90, 102, and 110), two of which (ORF^{V} 41 and 110) also included a RING finger domain, eight contained zinc finger RING-type domains (ORF^{V}s 10, 37, 56, 99, 100, 121, 122, 125), and two dUTPase-like domains (ORF^{V}s 28 and 78). Interestingly, ORF^{V} 75 showed similarities with ORFs 116/126, C-terminal of the White spot syndrome virus (WSSV), an OIE-listed disease affecting crustacean hosts. Ten genes were predicted to encode secreted proteins (ORF^{V}s 14, 16, 18, 38, 43, 52, 67, 77, 86, and 124) comprised ORF^{V} 43 resulting from the 2671 bp insertion, and 19 genes were predicted to encode membrane proteins (ORF^{V}s 17, 23, 26, 33, 40, 46, 53, 57, 60, 62, 66, 70, 74, 80, 83, 87, 91, 106, and 115), ten of which are thought to be putative membrane glycoproteins (ORF^{V}s 26, 33, 46, 57, 62, 66, 80, 83, 91, and 115). ORF^{V} 60 encodes a protein related to cellular chloride ion channel (MCLC). ORF^{V} 23

acquired a transmembrane helix region due to mutations with respect to the reference, but no N-glycosylation site was predicted. Several of the ORFs encoding putative membrane glycoproteins showed interesting mutations potentially involved in the higher virulence of the μVar genotype: ORF^V 91, ORF^V 80, ORF^V 62, and ORF^V 57 with the substitution of 10, 7, 3, 2 amino acids respectively. Finally, ORF^V 46, resulting from the 2671 bp insertion in $U_{L,}$ encodes for an additional putative membrane glycoprotein as compared to the reference genotype. All ORFs encoding IAPs and four of the eight ORFs encoding RING finger proteins presented changes in the polypeptide chain. However, while these mutations mainly concerned the substitution of one to two codons, ORF^Vs 10 and 37 showed deep transformation. In fact, the ORF^V 10 polypeptide chain was truncated for six codons because of a mutation inducing anticipation of the stop codon. With additional seven codon substitutions, this ORF showed high diversity with respect to the reference genotype. The second large deletion observed along the reference genome induced the loss of 37 codons in ORF^V 37 and the addition of 29 different codons until the next stop codon. The addition of 31 codons was also observed in ORF^V 124, which encodes a putative secreted protein. Lastly, the function of the 78 remaining ORFs remains unknown. However, the loss of 462, 101, 3, 17, and 43 codons in ORF^Vs 12, 34, 104, 118, and 123, and the addition of 39 and 41 codons in ORF^Vs 36 and 118, respectively, due to single nucleotide substitutions affecting the start or the stop codon or caused by the presence of the large indels, probably altered their primary function. About 72% of the 68 single nucleotide polymorphisms (SNPs) detected between variant A and variant B were situated in coding regions, inducing the substitution of one codon in 22 ORFs (ORF^Vs 4, 8, 10, 12, 13, 15, 18, 20, 22, 29, 33, 50, 51, 57, 59, 66, 67, 68, 69, 111, 118, and 119) and two codons in six ORF^Vs (ORFs 24, 79, 81, 91, 97, and 103). Finally, 57% of the ORFs with these mutations have an unknown function and 14% are transmembrane proteins. The ORFs encoding the Helicase/Primase Complex, DNA

Polymerase, and Replication Origin-binding Protein were also involved in one codon substitution between variant A and B.

Discussion

In the event of non-cultivable viruses or the absence of appropriate cell lines, studies of the virus genome are often a challenge. Such is the case for mollusc viruses, like OsHV-1 because of the unavailability of mollusc cell cultures (Yoshino *et al*., 2013). Moreover, whole-genome sequencing methods by cosmid/plasmid cloning are very time-consuming and the primer-walking method, developed on the basis of a reference sequence, may be unreliable when large indels or mutations in the primer target region are present. In order to sequence the whole genome of OsHV-1 µVar, we used an NGS technique with Illumina NextSeq500. We applied two different approaches according to the type of samples processed. We used crude homogenized soft tissue samples from infected individuals for the most rapid approach and gradient ultracentrifugation for nucleocapsid purification in the second approach. In the purification operations, the use of artificial sea water supplemented with TWEEN 20® (Sigma-Aldrich® Co.), a non-ionic detergent, during the first step of cell disruption gave the best results, probably owing to the maximum delivery of nucleocapsids thanks to improved the lysis of cell membranes. Both approaches had limitations, however. For example, the first suffered from high contamination with non-virus DNA and the second demanded technical expertise and specialized equipment, was time consuming, and with a restricted efficiency. Only with the use of both approaches we were able to obtain the complete genome sequence of µVar, combining deep coverage and distinctness of the results.

Though OsHV-1 µVar was found to be related to the OsHV-1 reference genotype, numerous and sometimes important mutations in coding regions may explain its higher virulence in young Pacific oysters. A core-set of genes necessary for viral DNA replication, and present in other viruses with long DNA genomes, was present also in OsHV-1 µVar. Unfortunately, we

were unable to determine the function of 56% of ORFs, which complicates interpretation of the differences in virulence observed between the µVar and the reference genomes. Among the deleted ORFs, only ORF^{R} 36 was thought to encode a membrane protein.

The same observation could be mentioned for the effect of the lighter mutations, such as codon substitution, deletion and insertion. The four ORFs encoding for putative BIR proteins, with a possible anti-apoptotic activity (Miller, 1999), showed interesting codon variations though they involve the substitution of no more than two codons. BIR genes are commonly found in invertebrate viruses, including *Ascoviridae* (Stasiak *et al*., 2000), *Asfaviridae* (Yáñez *et al*., 1995), *Baculoviridae* (Crook *et al*., 1993), *Iridoviridae* (Jakob *et al*., 2001), and *Poxviridae* (Afonso *et al*., 1999), and are believed to interfere with host defense against viral infections. The putative RING proteins demonstrated a relative stability insofar as the polypeptide chain was deeply changed in only two ORFs, implicating the probable loss of their initial function, while only two of the six others showed the substitution of one and two codons, respectively. Alphaherpesvirus proteins, including RING finger domains such as ICP0 in HSV-1 and Bovine herpesvirus 1 (BHV-1) are involved not only in viral transcription and productive infection activation but also in the inhibition of interferon transcription, which disables host antiviral response (Saira *et al*., 2009; Delboy & Nicola, 2011). They are also present in *Alloherpesviridae* such as Koi herpesvirus (KHV) (Aoki *et al*., 2007). Though their action in herpesviruses with invertebrate hosts is not yet completely understood, they are probably involved in virus virulence. The putative secreted proteins showed elevated stability, with only three of them displaying the substitution of only one codon. In contrast, the codon sequence of putative membrane glycoproteins differed massively to the reference genotype for some genes, such as ORF 91, as also observed by Martenot *et al*. (2013).

A common feature these proteins, present on the viral envelope, share is the critical role they play in virus entry into the host cell. The cytoplasmic membrane of eukaryotic cells serves as a barrier against invading viruses. In order to infect a cell, viruses must be capable of

transporting their genome and accessory proteins into the host cell, bypassing the barrier properties of the plasma membrane. Entry into the host cells always involves a step of binding to cell surface receptors, mediated by specific viral surface glycoproteins recognized by the host cell and followed by membrane fusion for herpesviruses such as OsHV-1. Fusion glycoproteins have been identified for most enveloped animal viruses (Bentz, 1993). However, this feature suggests that these molecular structures are probably also targeted by the innate immune system of molluscs, as observed in HCMV where two envelope glycoproteins are necessary for the activation of a Toll-like receptor (TLR2), involved in inflammatory cytokine responses to viral infections (Boehme *et al*., 2006) or during host response to HSV-1 where the activation of monocyte-derived dendritic cell is induced by the four essential viral glycoproteins, gB, gD, and gHgL (Reske *et al*., 2008). Changes in these glycoproteins could promote immune escape. Because these envelope viral glycoproteins are under high selection pressure, they usually have an elevated genetic diversity that plays an important role in the evolution of virus virulence (Vigerust and Shepherd, 2007). Among the glycoproteins of OsHV-1, and as a consequence of nucleotide substitutions, we observed in ORF^{V} 57 the addition of an N-glycosylation site with respect to the correspondent ORF^{R} 54 in the reference type, and the displacement of one of these sites in ORF^{V} 62 (ORF^{R} 59), and in ORF^{V} 91 (ORF^{R} 88). Furthermore, the 2671 bp insertion in U_L is responsible for the presence of four new ORFs encoding a supplementary membrane glycoprotein, in addition to a secreted protein and two proteins with unknown function. Since the differences between the reference and the µVar glycoproteins and other proteins are remarkable and numerous, it is not possible to incriminate a specific mutation as being responsible for the increased virulence of the µVar genotype. Interestingly, when we compare variant A with variant B, which are very closely related, we found that the set of genes involved in DNA replication, which share common functions among the herpesviruses, may be a suitable target for phylogenetical studies.

Conclusion

Our results demonstrated the suitability of the Illumina technique for sequencing the whole long-length genome of a dsDNA virus such as OsHV-1, by alignment on a reference related genome and assisted by software that allow the detection of insertions and the read assembly also from unpurified samples. The two OsHV-1 genotypes, reference and µVar, were found to be genetically closely related. However, the presence of a conspicuous number of nucleotide substitutions in numerous coding regions and the deletion or addition of different ORFs and of entire regions of the genome suggest that the two clusters (µVar on one hand and AVNV/OsHV-1 reference on the other), as reported in previous studies (Martenot *et al*., 2013; Mineur *et al*., 2015; Burioli *et al*., 2016), may have separated long ago. If such is the case, the increase in mortality events occuring since 2008 may not be due to an accrued virulence induced by a direct mutation of the reference genotype, but rather to an exogenous origin of the µVar genotype or to the mutation of a closely related variant of µVar that was present before 2008 in France. Whatever the case, the availability of the complete genome sequence of the OsHV-1 µVar will contribute to improving our knowledge of virulence factors in viruses with mollusc hosts.

Acknowledgements

We thank D. Vaur and all the Laboratory of Biology and Genetics of Cancer working team from the Centre François BACLESSE (Caen-France) for their valuable consulting during the use of the NGS technology platform and especially for the data analysis.

Batch	Sampling Site	Latitude	Longitude	Sampling Date	Origin	Ploidy	Viral load
Meuv	Meuvaines (France)	49°21'00.59"N	0°32'42.07"O	24-Jun-2010	Hatchery	Triploid	4.10^9 GU/50mg
Dung	Dungloe (Ireland)	54°56'36.33"N	8°23'49.42"O	5-Aug-2011	Hatchery	Triploid	2.10^9 GU/50mg
Géfo	Géfosse-Fontenay (France)	49°23'14.00"N	1° 5'53.30"O	22-Jun-2011	Charente (France)*	Diploid	3.10^9 GU/50mg
Blai	Blainville (France)	49° 3'25.73"N	1°36'36.49"O	Jun-2014	Charente (France)*	Diploid	4.10^7 GU/50mg

Table 1. Sample description.*natural collection.

Protocols			
A	**B**	**C**	**D**
Tissue disruption Ultra-Turrax®			
10 g. oyster soft tissues* + 140 mL PMSF 1mM			
15 mL ASW	15 mL ASW + 750 µL TWEEN 20	15 mL PBS	15 mL DW
Centrifugation			
250 x g 30 min 1000 x g 30 min 4000 x g 30 min			
Filtration			
5 µm 0.45 µm 0.22 µm			
Each sample is divided in two aliquots			
Ultracentrifugation 80,000 x g 1 h			

A	**A'**	**B**	**B'**	**C**	**C'**	**D**	**D'**
DNAse**	Gradient **	DNAse**	Gradient **	DNAse**	Gradient **	DNAse**	Gradient **

Table 2. Description of the compared pretreatments used to improve the viral DNA/exogenous DNA ratio.
ASW: Artificial Sea Water (NaCl 35 ‰); PBS: Phosphate-Buffered Saline (NaCl 8‰); DW : Deionized Water ; *, quantifiaction by OsHV-1 qPCR ; **, quantifi

Description	Target region in AY509253	Sequence	Amplicon length (bp)
Ins1-F	Insertion	5' ATCTGCAACTGTCTGCAACG 3'	816
Ins1-R	nucleotide 1655	5' CAGTATGCATCACCACAATCG 3'	
Del1-F	Deletion	5' AATTCAACGGGAAACAGACC 3'	1313
Del1-R	nucleotide 17,707 to 19,092	5' TCTCCATTTCCTTGGACTGC 3'	
Del2-F	Deletion	5' ATACGATGCGTCGGTAGAGC 3'	458
Del2-R	nucleotide 52,253 to 52,858	5' AGAGCGATGGCAAAATTACG 3'	
Ins2-F		5' AACCGCGAAAGAAAAGATCC 3'	913
Ins2-R		5' CCACGGGTTAATCCATTTCC 3'	
Ins3-F		5' TGCTGGGAGTATCACTTTGC 3'	879
Ins3-R	Insertion	5' GCCGGTATATTCCTTGTTGC 3'	
Ins4-F	nucleotide 60,740	5' CAACAAGGAATATACCGGCAG 3'	418
Ins4-R		5' ATATGTCATGAAAGTCGGCG 3'	
Ins5-F		5' GACATGGGTAAAACATCAGAGG 3'	1054
Ins5-R		5' CGGAAAGGAAAAGTCTGTGG 3'	
Del3-F	Deletion	5' ACATTTCATCATGCCCAAGG 3'	926
Del3-R	nucleotide 67,973 to 68,572	5' TTCCGGGATAAATAGCATGG 3'	
Del4-F	Deletion	5' ACATGTTCATCTGCCACAGG 3'	1250
Del4-R	nucleotide 93,120 to 96,669	5' AAACCACCTGCCATACTTGG 3'	
Del5-F	Deletion	5' TCTTGGGAATGGTGAAGAGC 3'	772
Del5-R	nucleotide 175,018 to 175,743	5' TTTCCAATTCCGTCTTCTCG 3'	
Ins6-F	Insertion	5' TTACTGGCAGCAGCAATACC 3'	582
Ins6-R	nucleotide 181,357	5' CATCCCCGTGTTAAATCTCC 3'	

Table 3. Primers used for the validation of indels observed in OsHV-1 µVar

ORF OsHV-1 µVar	Length (bp)	Length (codons)	Function/Family7Domain	ORF OsHV-1 reference	Length (bp)	Nucleotide seq. Similarity	Codon substitutions	Acquired or lost codons
ORF^V 1	447	148	unknown	ORF^R 1	"	100.0%	0	
ORF^V 2	504	167	unknown	ORF^R 2	"	99.8%	1	
ORF^V 3	768	255	unknown	ORF^R 3	765	99.0%	2	+ 1
ORF^V 4	1050	349	unknown	ORF^R 4	"	99.5%	5	
ORF^V 5	738	245	unknown	ORF^R 5	disrupted	-	-	
ORF^V 6	378	125	unknown					
ORF^V 7	2031	676	unknown	ORF^R 6	"	100%	0	
ORF^V 8	3546	1181	component of helicase/primase complex, helicase	ORF^R 7	"	99.7%	7	
ORF^V 9	960	319	unknown	ORF^R 8	"	100%	0	
ORF^V 10	1761	586	Zinc-finger, Ring type	ORF^R 9	1779	98.4%	7	- 6
ORF^V 11	1029	342	unknown	ORF^R 10	"	99.9%	0	
ORF^V 12	1902	633	unknown	ORF^R 11	3288	57.6%	8	- 462
ORF^V 13	588	195	unknown	ORF^R 12	"	99.8%	1	
ORF^V 14	318	105	secreted	ORF^R 13	"	99.7%	1	
ORF^V 15	585	194	unknown	ORF^R 14	"	99.8%	1	
ORF^V 16	645	214	secreted	ORF^R 15	"	99.8%	1	
ORF^V 17	231	76	membrane protein	ORF^R 16	"	100%	0	
ORF^V 18	348	115	secreted	ORF^R 17	"	100%	0	
ORF^V 19	282	93	unknown	ORF^R 18	"	99.6%	1	
ORF^V 20	1209	402	unknown	ORF^R 19	"	99.7%	3	
ORF^V 21	1740	579	Ribonucleotide reductase small subunit	ORF^R 20	"	99.9%	1	
ORF^V 22	2955	984	unknown	ORF^R 21	"	99.8%	5	
ORF^V 23	4899	1632	transmembrane protein, 1 helix	ORF^R 22	"	99.8%	5	
ORF^V 24	3819	1272	unknown	ORF^R 23	"	99.8%	8	
ORF^V 25	1134	377	unknown	ORF^R 24	"	99.9%	1	
ORF^V 26	666	221	transmembrane glycoprotein, 1 helix	ORF^R 25	"	100%	0	
ORF^V 27	1188	395	unknown	ORF^R 26	"	99.7%	3	
ORF^V 28	801	266	dUTPase-like	ORF^R 27	"	99.9%	0	
ORF^V 29	2562	853	unknown	ORF^R 28	"	99.8%	3	
ORF^V 30	606	201	unknown	ORF^R 29	"	99.7%	2	
ORF^V 31	747	248	Herpes_UL92	ORF^R 30	"	99.9%	0	
ORF^V 32	558	185	unknown	ORF^R 31	"	99.8%	1	
ORF^V 33	1659	552	transmembrane glycoprotein, 1 helix	ORF^R 32	disrupted	-	-	
ORF^V 34	588	195	unknown	ORF^R 33	891	65.8%	1	- 101
ORF^V 35	375	124	unknown	ORF^R 34	"	100%	0	
ORF^V 36	591	196	unknown	ORF^R 35	474	80.2%	0	+ 39
-	-	-	-	ORF^R 36	228	deleted	-	
-	-	-	-	ORF^R 37	231	deleted	-	
ORF^V 37	558	185	Zinc-finger, Ring type	ORF^R 38	582	82.2%	2	- 37 + 29
ORF^V 38	585	194	secreted	ORF^R 39	"	99.7%	1	
ORF^V 39	1728	575	unknown	ORF^R 40	"	99.9%	1	
ORF^V 40	2922	973	transmembrane protein, 1 helix	ORF^R 41	2919	99.6%	7	+ 1
ORF^V 41	1095	364	Zinc-finger, Ring type, BIR domain	ORF^R 42	"	99.7%	2	
ORF^V 42	612	203	unknown	ORF^R 43	"	99.8%	0	
ORF^V 43	525	174	secreted	-	-	-	-	
ORF^V 44	348	115	unknown	-	-	-	-	
ORF^V 45	474	157	unknown	-	-	-	-	
ORF^V 46	981	326	transmembrane glycoprotein, 1 helix	-	-	-	-	
ORF^V 47	927	308	Deoxynucleoside kinase domain	ORF^R 44	"	99.7%	1	
ORF^V 48	726	241	unknown	ORF^R 45	"	100%	0	
ORF^V 49	648	215	unknown	ORF^R 46	"	99.8%	0	
ORF^V 50	4239	1412	unknown	ORF^R 47	"	99.9%	3	
-	-	-	-	ORF^R 48	483	deleted	-	
ORF^V 51	3417	1138	unknown	ORF^R 49	"	99.9%	3	
ORF^V 52	1302	433	secreted	ORF^R 50	disrupted	-	-	
ORF^V 53	507	168	transmembrane protein, 1 helix					
ORF^V 54	2508	835	Ribonucleotide reductase large subunit	ORF^R 51	"	99.9%	2	
ORF^V 55	543	180	unknown	ORF^R 52	"	99.8%	0	
ORF^V 56	1548	515	Zinc-finger, Ring type	ORF^R 53	"	100%	0	
ORF^V 57	2424	807	transmembrane glycoprotein, 1 helix	ORF^R 54	"	99.8%	2	
ORF^V 58	420	139	unknown	ORF^R 55	"	100%	0	
ORF^V 59	849	282	unknown	ORF^R 56	"	99.5%	3	
ORF^V 60	951	316	transmembrane protein, 3 helix, MCLC	ORF^R 57	"	99.8%	2	
ORF^V 61	1566	521	unknown	ORF^R 58	"	99.9%	1	
ORF^V 62	3243	1080	transmembrane glycoprotein, 1 helix	ORF^R 59	"	99.8%	3	
ORF^V 63	1236	411	unknown	ORF^R 60	"	100%	0	

ORF^{V} 64	1737	578	unknown	ORF^{R} 61	"	99.9%	1	
-	-	-	-	ORF^{R} 62	1802	deleted	-	
-	-	-	-	ORF^{R} 63	1865	deleted	-	
ORF^{V} 65	1197	398	RNA-ligase_T4 phage	ORF^{R} 64	"	99.8%	1	
ORF^{V} 66	1263	420	transmembrane glycoprotein, 1 helix	ORF^{R} 65	disrupted	-	-	
ORF^{V} 67	579	192	secreted					
ORF^{V} 68	3393	1130	DNA primase, small subunit	ORF^{R} 66	"	99.8%	1	
ORF^{V} 69	1791	596	P-loop NTPase, helicase	ORF^{R} 67	"	99.8%	2	
ORF^{V} 70	2082	693	transmembrane protein, 1 helix	ORF^{R} 68	"	100%	0	
ORF^{V} 71	1392	463	unknown	ORF^{R} 69	"	99.8%	1	
ORF^{V} 72	603	200	unknown	ORF^{R} 70	"	100%	0	
ORF^{V} 73	1362	453	unknown	ORF^{R} 71	"	99.8%	1	
ORF^{V} 74	567	188	transmembrane protein, 1 helix	ORF^{R} 72	"	100%	0	
ORF^{V} 75	627	208	White spot syndrome virus (WSSV), Orf116/126, C	ORF^{R} 73	disrupted	-	-	
ORF^{V} 76	678	225	unknown					
ORF^{V} 77	357	118	secreted	ORF^{R} 74	"	100%	0	
ORF^{V} 78	711	236	dUTPase-like	ORF^{R} 75	"	100%	0	
ORF^{V} 79	2037	678	unknown	ORF^{R} 76	"	100%	0	
ORF^{V} 80	3795	1264	transmembrane glycoprotein, 2 helix	ORF^{R} 77	"	99.7%	7	
ORF^{V} 81	3456	1151	unknown	ORF^{R} 78	"	99.5%	12	
ORF^{V} 82	441	146	unknown	ORF^{R} 79	"	99.8%	1	
ORF^{V} 83	351	116	transmembrane glycoprotein, 1 helix	ORF^{R} 80	"	99.7%	0	
ORF^{V} 84	642	213	unknown	ORF^{R} 81	"	99.8%	1	
ORF^{V} 85	891	296	unknown	ORF^{R} 82	"	99.8%	1	
ORF^{V} 86	1107	368	secreted	ORF^{R} 83	"	99.9%	0	
ORF^{V} 87	357	118	transmembrane protein, 1 helix	ORF^{R} 84	"	99.7%	1	
ORF^{V} 88	2004	667	unknown	ORF^{R} 85	"	99.9%	2	
ORF^{V} 89	408	135	unknown	ORF^{R} 86	"	100%	0	
ORF^{V} 90	513	170	BIR repeat	ORF^{R} 87	"	99.8%	1	
ORF^{V} 91	2247	748	transmembrane glycoprotein, 1 helix	ORF^{R} 88	"	99.6%	10	
ORF^{V} 92	735	244	unknown	ORF^{R} 89	"	99.9%	1	
ORF^{V} 93	885	294	unknown	ORF^{R} 90	"	99.7%	3	
ORF^{V} 94	1083	360	unknown	ORF^{R} 91	"	99.8%	0	
ORF^{V} 95	690	229	unknown	ORF^{R} 92	"	100%	0	
ORF^{V} 96	1215	404	unknown	ORF^{R} 93	"	100%	0	
ORF^{V} 97	1044	347	unknown	ORF^{R} 94	"	99.8%	2	
ORF^{V} 98	933	310	Exonuclease	ORF^{R} 95	"	99.8%	1	
ORF^{V} 99	723	240	Zinc-finger, Ring type	ORF^{R} 96	"	99.9%	0	
ORF^{V} 100	546	181	Zinc-finger, Ring type	ORF^{R} 97	"	99.6%	1	
ORF^{V} 101	1755	584	unknown	ORF^{R} 98	"	99.8%	2	
ORF^{V} 102	753	250	BIR repeat	ORF^{R} 99	"	99.6%	2	
ORF^{V} 103	5637	1878	Catalytic subunit DNA polymerase, family B	ORF^{R} 100	"	99.8%	9	
ORF^{V} 104	630	209	unknown	ORF^{R} 101	"	100%	0	
ORF^{V} 105	2289	762	unknown	ORF^{R} 102	"	99.9%	1	
ORF^{V} 106	1272	423	transmembrane protein, 4 helix	ORF^{R} 103	"	99.8%	0	
ORF^{V} 107	3603	1200	unknown	ORF^{R} 104	3612	99.7%	0	- 3
ORF^{V} 108	732	243	unknown	ORF^{R} 105	disrupted	-	-	
ORF^{V} 109	486	161	unknown					
ORF^{V} 110	1398	465	Zinc-finger, Ring type, BIR domain	ORF^{R} 106	"	99.6%	1	
ORF^{V} 111	2070	689	unknown	ORF^{R} 107	"	99.8%	3	
ORF^{V} 112	813	270	unknown	ORF^{R} 108	"	99.8%	2	
ORF^{V} 113	2625	874	Large subunit DNA-Packing terminase	ORF^{R} 109	"	100%	0	
ORF^{V} 114	786	261	unknown	ORF^{R} 110	"	100%	0	
ORF^{V} 115	870	289	transmembrane glycoprotein, 5 helix	ORF^{R} 111	"	100%	0	
ORF^{V} 116	1389	462	unknown	ORF^{R} 112	"	99.9%	2	
ORF^{V} 117	957	318	unknown	ORF^{R} 113	"	99.8%	2	
ORF^{V} 118	969	322	unknown	ORF^{R} 114	1485	61.6%	0	- 17 + 41
ORF^{V} 119	762	253	Replication origin-binding protein	ORF^{R} 115	"	99.9%	0	
ORF^{V} 120	771	256	unknown	ORF^{R} 116	"	99.6%	3	
ORF^{V} 121	1116	371	Zinc-finger, Ring type	ORF^{R} 117	"	99.8%	2	
ORF^{V} 122	669	222	Zinc-finger, Ring type	ORF^{R} 118	"	99.7%	0	
ORF^{V} 123	450	149	unknown	ORF^{R} 119	579	77.1%	2	- 43
ORF^{V} 124	324	107	secreted	ORF^{R} 120	231	71.8%	0	+ 31
ORF^{V} 125	648	215	Zinc-finger, Ring type	ORF^{R} 121	"	100%	0	
ORF^{V} 126	1155	384	unknown	ORF^{R} 122	"	99.6%	5	
ORF^{V} 127	912	303	unknown	ORF^{R} 123	"	99.9%	0	
ORF^{V} 128	1425	474	unknown	ORF^{R} 124	"	99.9%	1	

Table 4. OsHV-1 µVar ORF homologues of OsHV-1 reference, comparison and putative function.

Afonso C.L., Tulman E.R., Lu Z., Oma E., Kutish G.F., Rock D.L., 1999. The genome of *Malanoplus sanguinipes* entomopoxvirus. J. Virol., 73: 533-552. PMID: PMC103860.

Aoki T., Hirono I., Kurokawa K., Fukuda H., Nahary R., Eldar A., Davison A.J., Waltzek T.B., Bercovier H., Hedrick R.P., 2007. Genome sequences of three Koi herpesviruses isolates representing the expanding distribution of an emerging disease threatening Koi and Common carp worldwide. J. Virol., 81(10): 5058-5065. doi: 10.1128/JVI.00146-07.

Barzon L., Lavezzo E., Militello V., Toppo S., Palu G., 2011. Applications of next-generation sequencing technologies to diagnostic virology. Int. J. Mol. Sci., 12: 7861-7884. doi: 10.3390/ijms12117861.

Batista F.M., López-Sanmartín M., Grade A., Morgado I., Valente M., Navas J.I., Power D.M., Ruano F., 2015. Sequence variation in Ostreid herpesvirus 1 microvar isolates detected in dying and asymptomatic *Crassostrea angulata* adults in the Iberian Peninsula: Insights into viral origin and spread. Aquaculture, 435: 43-51. doi: 10.1016/j.aquaculture.2014.09.016.

Bentley D.R., Balasubramanian S., Swerdlow H.P., Smith G.P., Milton J., Brown C.G., *et al*., 2008. Accurate whole genome sequencing using reversible terminator chemistry. Nature, 456: 53-59. doi: 10.1038/nature07517.

Bentz J., 1993. Viral Fusion Mechanisms. Boca Raton, FL: CRC Press. 529 pp.

Boehme K.W., Guerrero M., Compton T., 2006. Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J. Immunol., 84: 12292-12299. PMID: 17082626.

Burge C.A., Griffin F.J., Friedman C.S., 2006. Mortality and herpevirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. Dis. Aquat. Org., 72: 31-43. doi: 10.3354/dao072031.

Burioli E.A.V., Prearo M., Riina M.V., Bona M.C., Fioravanti M.L., Arcangeli G., Houssin M., 2016. Ostreid herpesvirus type 1 genomic diversity in wild populations of Pacific oyster *Crassostrea gigas* from Italian coasts. J. Invertebr. Pathol., 137: 71-83. doi: 10.1016/j.jip.2016.05.004.

Chartrand P., Crumpacker C.S., Schaffer P.A., Wilkie M.N., 1980. Physical and genetic analysis of the herpes simplex DNA polymerase locus. Virology, 103: 311-326. PMID: 6247818.

Cochennec-Laureau N., Baud J.P., Bedier E., Boudry P., Huvet A., Nicolas J.L., Pépin J.F., Petton B., 2009. "Journées Surmotalités des huitres creuses, *Crassostrea gigas*" du programme P7 "Aquaculture Durable" des 8-9 décembre 2009. htpp://sd2.archivehost.com/membres/up/21415313303356231O/IFREMER_8_et_9_decembre_2009.pdf

Cohen G.H., 1972. Ribonucleotide reductase activity of synchronized KB cells infected with herpes simplex virus. J. Virol., 9: 408-418. PMCID: PMC356313.

Crook N.E., Clem R.J., Miller L.K., 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J. Virol., 67: 2168-2174. PMCID: PMC240327.

Crute J.J., Tsurumi T., Zhut L., Wellert S.K., Olivot D.P., Challbergt D.M., Mocarski S.E., Lehman I.R., 1989. Biochemistry Herpes simplex virus 1 helicase-primase: A complex of three herpes-encoded genes. Proc. Nati. Acad. Sci. USA, 86: 2186-2189. PMCID: PMC286876

Dai X., Yu X., Gong H., Jiang X., Abenes G., Liu H., Shivaloti S., Britt W.J., Zhu H., Liu F., Zhou Z.H., 2013. The smallest capsid protein mediates binding of the essential tegument protein pp150 to stabilize DNA-containing capsids in human cytomegalovirus. PloS Patho., 9(8): e1003525. doi: 10.1371/journal.ppat.1003525.

Dai X., Zhou Z.H., 2014. Purification of Herpesvirus Virions and Capsids. Bio-protocol, 4(15), Aug 05, 2014. http://www.bio-protocol.org/e1193. PMCID: PMC5087969.

Davison A.J., 2002. Evolution of the herpesviruses. Vet. Microbiol., 86: 69-88. doi: 10.1016/S0378-1135(01)00492-8.

Davison A.J., Davison M.D, 1995. Identification of Structural Proteins of Channel Catfish Virus by Mass Spectrometry. Virology, 1035-1043.

Davison A.J., Dolan A., Akter P., Addison C., Dargan D.J., Alcendor D.J., McGeoch D.J., Hayward G.S., 2003. The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. J. Gen. Virol., 84(1): 17–28. doi: 10.1099/vir.0.18606-0.

Davison A.J., Eberle R., Ehlers B., Hayward G.S., McGeoch D.J., Minson A.C., Pellett P.E., Roizman B., Studdert M.J., Thiry E., 2009. The Order *Herpesvirales*. Arch. Virol., 154(19): 171-177. doi: 10.1007/s00705-008-0278-4.

Davison A.J., Trus B.L., Cheng N., Steven A.C., Watson M.S., Cunningham C., Le Deuff R.M., Renault T., 2005. A novel class of herpesvirus with bivalve hosts. J. Gen. Virol., 86(1): 41-53. doi: 10.1099/vir.0.80382-0.

Delboy M.G., Nicola A.V., 2011. A pre-immediate-early role for tegument ICP0 in the proteasome-dependent entry of herpes simplex virus. J. Virol., 85: 5910-5918. doi: 10.1128/JVI.00267-11.

Dubbs D.R., Kit S., 1964. Mutant strains of herpes simplex virus deficient in thymidine kinase activity. Virology, 22: 493-502. PMCID: PMC282940.

Dundon W.G., Arzul I., Omnes E., Robert M., Magnabosco C., Zambon M., Gennari L., Toffan A., Terregino C., Capua I., Arcangeli G., 2011. Detection of Type 1 Ostreid Herpes variant (OsHV-1 µVar) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. Aquaculture, 314(1-4): 49-52. doi: 10.1016/j.aquaculture.2011.02.005.

Dutia B.M., 1983. Ribonucleotide reductase induced by herpes simplex virus has a virus specified constituent. J. Gen. Virol., 65: 513-521. doi: 10.1099/0022-1317-64-3-513.

EFSA, European Food Safety Authority, 2015. EFSA Panel on Animal Health and Welfare (AHAW): Scientific opinion on oyster mortality. EFSA Journal, 13(6): 4122-4180. doi: 10.2903/j.efsa.2015.4122.

Elias P., O'Donnell M.E., Mocarski E.S., Lehman I.R., 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Nat. Acad. Sci. USA, 83: 6322-6326. PMCID: PMC386495.

FAO, Food and Agriculture Organisation of the United States, 2014. Fisheries and Aquaculture Department, Dateset Global Aquaculture production. On-Line Query.

Farley C.A., Banfield W.G., Kasnic Jr. G., Foster W.S., 1972. Oyster herpes-type virus. Science, 178: 759–760.

Friedman C.S., Estes R.M., Stokes N.A, Burges C.A., Hargove J.S., Barber B.J, Elston R.A., Burreson E.M., Reece K.S., 2005. Herpes virus in juvenile Pacific oysters *Crassostrea*

gigas from Tomales Bay, California, coincides with summer mortality episodes. Dis. Aquat. Org., 63: 33-41. doi: 10.3354/dao063033.

Gire S.K., Goba A., Andersen K.G., Sealfon R.S., Park D.J., Kanneh L., et al., 2014. Genomic surveillance to elucidate Ebola virus origin and transmission during the 2014 outbreak. Science, 345(6202): 1369-1372. doi: 10.1126/science.1259657.

Gittenberger A., Voorbergen-Laarman M.A., Engelsma M.Y., 2016. Ostreid herpesvirus OsHV-1 µVar in Pacific oysters *Crassostrea gigas* (Thunberg, 1793) of the Wadden Sea, a UNESCO world heritage site. J. Fish Dis., 39(1): 105-109. doi: 10.1111/jfd.12332.

Goodwin S., McPherson J.D., McCombie W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics, 17: 333–351. doi:10.1038/nrg.2016.49.

Grijalva-Chon J.M., Castro-Longoria R., Ramos-Paredes J., Enríquez-Espinoza T.L., Mendoza-Cano F., 2013. Detection of a new OsHV-1 DNA strain in the healthy Pacific oyster, *Crassostrea gigas* (Thunberg) from the Gulf of California. J. Fish Dis., 36: 965-968. doi: 10.1111/jfd.12028.

Hayward G.S., Jacob R.J., Wadsworth S.C., Roizman B., 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc. Natl. Acad. Sci. USA, 72: 4243-4247. PMCID: PMC388696.

Hine P.M., Wesney B., Hay B.E., 1992. Herpesviruses associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*. Dis. Aquat. Org., 12: 135-142.

Hwang J.Y., Park J.J., Yu H.J., Hur Y.B., Arzul I., Couraleau Y., Park M.A., 2013. Ostreid herpesvirus 1 in Pacific oyster *Crassostrea gigas* larvae (Thunberg) in Korea. J. Fish Dis., 36(11): 369-372. doi: 10.1111/jfd.12093.

ICTV, International Committee on Taxonomy of Viruses, 2015. Virus Taxonomy, 2015 Release. EC 47, London, UK, July 2015. http://www.ictvonline.org/virustaxonomy.asp.

Jakob N.J., Müller K., Bahr U., Darai G., 2001. Analysis of the first complete DNA sequence of an invertebrate iridovirus: coding strategy of the genome of *Chilo* iridescent virus. Virology, 286: 182-196. doi:10.1006/viro.2001.0963.

Jenkins C., Hick P., Gabor M., Spiers Z., Fell S.A., Gu X., Read A., Go J., Dove M., O'Connor W., Kirkland P.D., Frances J., 2013. Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* in Pacific oysters in Australia. Dis. Aquat. Organ. 105(2): 109-126. doi: 10.3354/dao02623.

Keeling S.E., Brosnahan C.L., Williams R., Gias E., Hannah M., Bueno R., McDonald W.L., Johnston C., 2014. New Zealand juvenile oyster mortality associated with Ostreid herpesvirus 1 – an opportunistic longitudinal study. Dis. Aquat. Organ., 109: 231-239. doi: 10.3354/dao02735.

Kieff E.D., Bachenheimer S.L., Roizman B., 1971. Size, composition and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. 8: 125-132. PMCID: PMC356223.

Langmead B., Salzberg S., 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods, 9: 357–359. doi:10.1038/nmeth.1923.

Le Deuff R.M., Renault T., 1999. Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. J. Gen.Virol., 80: 1317-1322. doi: 10.1099/0022-1317-80-5-1317.

Li Z., Bai X., Ruparel H., Kim S, Turro N.J., Ju J., 2003. A photocleavable fluorescent nucleotide for DNA sequencing and analysis. Proc. Natl. Acad. Sci., USA, 100: 414-419. doi: 10.1073/pnas.242729199.

Loman N.J., Constantinidou C., Christner M., Rohde H., Chan J.Z., Quick J., et al., 2013. A culture-independent sequence-based metagenomics approach to the investigation of an outbreak of Shiga-toxigenic *Escherichia coli* 0104:H4. JAMA, 309(14): 1502-1510. doi: 10.1001/jama.2013.3231.

Lynch S.A., Carlsson J., Reilly A.O., Cotter O., Culloty S.C., 2012. A previously undescribed Ostreid herpes virus 1 (OsHV-1) genotype detected in the Pacific oyster, *Crassostrea gigas*, in Ireland. Parasitology, 139(12): 1526-1532. doi: 10.1017/S0031182012000881.

Martenot C., Fourour S., Oden E., Jouaux A., Travaillé E., Malas J.P., Houssin M., 2012. Detection of the OsHV-1 µVar in the Pacific oyster *Crassostrea gigas* before 2008 in France and description of two new microvariants of the Ostreid Herpesvirus (OsHV-1). Aquaculture, 338-341: 293-296. doi: 10.1016/j.aquaculture.2011.12.030.

Martenot C., Oden E., Travaillé E., Malas J.P., Houssin M., 2010. Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crasssotrea gigas*. J. Virol. Methods, 170: 86-89. doi:10.1016/j.jviromet.2010.09.003.

Martenot C., Oden E., Travaillé E., Malas J.P., Houssin M., 2011. Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. Virus Res., 160: 25-31. doi: 10.1016/j.virusres.2011.04.012.

Martenot C., Travaillé E., Lethuillier O., Lelong C., Houssin M., 2013. Genome exploration of six variants of the Ostreid Herpesvirus 1 andcharacterization of large deletion in OsHV-1µVar specimens. Virus Res., 178: 462– 470. doi: 10.1016/j.virusres.2013.08.006.

Miller L.K., 1999. An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell. Biol., 9: 323-328. doi: 10.1016/S0962-8924(99)01609-8.

Mineur F., Provan J., Arnott G., 2015. Phylogeographical analyses of shellfish viruses: inferring a geographical origin for ostreid herpesvirus OsHV-1 (*Malacoherpesviridae).* Mar. Biol., 162: 181-192. doi: 10.1007/s00227-014-2566-8.

Minson A.C., Davison A., Eberle R., Desrosiers R.C., Fleckenstein B., McGeoch D.J., Pellet P.E., Roizman B., Studdert M.J., 2000. Family Herpesviridae. In: Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses, Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 203–225.

Mitchell A., Chang H.Y., Daugherty L., Fraser M., Hunter S., Lopez R., et al., 2015. The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res., 43: 213-221. doi: 10.1093/nar/gku1243.

Morrissey T., McCleary S., Collins E., Henshilwood K., Cheslett D., 2015. An investigation of Ostreid Herpes Virus microvariants found in *Crassostrea gigas* oyster producing bays in Ireland. Aquaculture, 442: 86-92. doi: 10.1016/j.aquaculture.2015.02.026.

Mortensen S., Strand Å., Bodvin T., Alfjorden A., Skår C.K., Jelmert A., Aspán A., Saelemyr L., Naustvoll L.J., Albertsen J., 2016. Summer mortalities and detection of Ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway. Dis. Aquat. Organ., 117(3): 171-176. doi: 10.3354/dao02944.

Nicolas J.L., Comps M., Cochennec N., 1992. Herpes-like virus infecting Pacific-oyster larvae, *Crassostrea gigas*. Bull. Eur. Ass. Fish Pathol., 12(1): 11-13.

OIE, Office International des Epizooties, 2014. Infection with Ostreid herpesvirus 1 microvariants (May 2014 version). Manual of Diagnostic for Aquatic Animals 2015. Chap. 2.4.5. http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_ostreid_herpesvirus_1.htm.

Omasits U., Ahrens C.H., Müller S., Wollscheid B., 2014. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics, 30(6): 884-886. doi: 10.1093/bioinformatics/btt607.

Paul-Pont I., Evans O., Dhand N., Rubio A., Coad P., Whittington R., 2014. Descriptive epidemiology of mass mortality due to *Ostreid herpesvirus-1* (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas)* in the Hawkesbury River estuary, Australia. Aquaculture, 422-423: 146-159. doi: 10.1016/j.aquaculture.2013.12.009.

Peeler E.J., Reese R.A., Cheslett D.L., Geoghegan F., Power A., Thrush M.A., 2009. Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 µVar in the Republic of Ireland in 2009. Prev. Vet. Med., 105(1-2): 136-143. doi: 10.1016/j.prevetmed.2012.02.001.

Petersen T.N., Brunak S., von Heijne G., Nielsen H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods, 8: 785-786. doi:10.1038/nmeth.1701.

Ren W., Chen H., Renault T., Cai Y., Bai C., Wang C., Huang J., 2013. Complete genome sequence of Acute Viral Necrosis Virus associated with massive mortality outbreaks in the Chinese scallop, *Chlamys farreri*. Vir. J., 10: 110-117. doi: 10.1186/1743-422X-10-110.

Renault T., Allain G., Arzul I., Chollet B., Cobret L., De Decker S., et al., 2009. Summer mortality outbreaks of French Pacific Oyster, *Crassostrea gigas*, in 2008: research and detection of pathogens. Dis. Fish and Shellfish, 14th International Conference of the EAFP. Sept 14-19 Prague, Czeck Republic. abstract O: 112-127.

Renault T., Cochennec N., Le Deuff R.M., Chollet B., 1994a. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. Bull. Eur. Ass. Fish Pathol., 14(2): 64-66.

Renault T., Le Deuff R.M., Cochennec N., Maffart P., 1994b. Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France – Comparative study. Rev. Med. Vet., 145(10): 735-742.

Renault T., Moreau P., Faury N., Pépin J.F., Segarra A., Webb S., 2012. Analysis of clinical Ostreid Herpevirus 1 (*Malacoherpesviridae*) specimens by sequencing amplified fragments from three virus genome areas. J. Virol., 86(10): 5942-5947. doi: 10.1128/JVI.06534-11.

Reske A., Pollara G., Krummenacher C., Katz D.R., Chain B.M., 2008. Glycoprotein-Dependent and TLR2-Independent Innate Immune Recognition of Herpes Simplex Virus-1 by Dendritic Cells. J. Immunol., 180: 7525-7536. doi: 10.4049/jimmunol.180.11.7525.

Rizk G., Gouin A., Chikhi R., Lemaitre C., 2014. MindTheGap: integrated detection and assembly of short and long insertions. Bioinformatics, 30(24): 3451-3457. doi: 10.1093/bioinformatics/btu545.

Robinson J.T., Thorvaldsdóttir H., Winckler W., Guttman M., Lander E.S., Getz G., Mesirov J.P., 2011. Integrative Genomics Viewer. Nature Biotechnol., 29: 24–26. doi:10.1038/nbt.1754.

Roque A., Carrasco N., Andree K.B., Lacuesta B., Elandaloussi L., Gairin I., Rodgers C.J., Furones M.D., 2012. First report of OsHV-1 microvar in Pacific oyster (*Crassostrea gigas)* cultured in Spain. Aquaculture 324-325: 303-306. doi: 10.1016/j.aquaculture.2011.10.018.

Saira K, Zhou Y., Jones C., 2009. The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) associates with interferon regulatory factor 7 (IRF7), and consequently inhibits beta interferon promoter activity. J. Virol., 83(8): 3977–3981. doi: 10.1128/JVI.02400-08.

Savin K.W., Cocks B.G., Wong F., Sawbridge T., Cogan N., Savage D., Warner S., 2010. A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. Virol. J., 7: 308-316. doi: 10.1186/1743-422X-7-308.

Segarra A., Pépin J.F., Arzul I., Morga B., Faury N., Renault T., 2010. Detection and description of a particular Ostreid Herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res., 153 (1): 92–99. doi: 10.1016/j.virusres.2010.07.011.

Shimahara Y., Kurita J., Kiryu I., Nishioka T., Yuasa K., Kawana M., Kamaishi T., Oseko N.,2012. Surveillance of type 1 Ostreid Herpesvirus (OsHV-1) variants in Japan. Fish Pathol., 47(4): 129-136. doi: 10.3147/jsfp.47.129.

Silber R., Malathi V.G., Hurwitz J., 1972. Purification and Properties of Bacteriophage T4-Induced RNA Ligase. Proc. Nat. Acad. Sci. USA, 69: 3009-3013. PMCID: PMC389696.

Stasiak K., Demattei M.V., Federici B.A., Bigot Y., 2000. Phylogenetic position of the *Diadromus pulchellus* ascovirus DNA polymerase among viruses with large double-stranded DNA genomes. J. Gen. Virol., 81: 3059-3072. doi: 10.1099/0022-1317-81-12-3059.

Tan J., Lancaster M., Hyatt A., van Driel R., Wong F., Warner S., 2008. Purification of a herpes-like virus from abalone (*Haliotis* spp.) with ganglioneuritis and detection by transmission electron microscopy. J. Virol. Methods, 149: 338-341. doi: 10.1016/j.jviromet.2007.12.019.

Thorvaldsdóttir H., Robinson J.T., Mesirov J.P., 2013. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinfo., 14: 178-192. doi: 10.1093/bib/bbs017.

Vasquez-Yeomans R., Caceres-Martinez J., Figueras-Huerta A., 2004. Herpes-like virus associated with eroded gills of the Pacific oyster *Crassostrea gigas* in Mexico. J. Shellfish Res., 23: 417-419. doi: 10.3354/dao02189.

Vigerust D.J., Shepherd V.L., 2007. Virus glycosylation: role in virulence and immune interactions. Trends microbiol., 15: 211-218. doi: 10.1016/j.tim.2007.03.003.

Wagner M.J., Summer W.C., 1978. Structure of the joint region and the termini of the DNA of herpes simples virus type 1. J. Virol., 27: 374-387.

Weller S.K., Spadaro A., Schaffer J.E., Murray A.W., Maxam A.M., Schaffer P.A., 1985. Cloning, sequencing, and functional analysis of Ori_L, a herpes simplex virus type 1 origin of DNA replication. Mol. Cell. Biol., 5: 930-942. doi: 10.1128/MCB.5.5.930.

Weststrate M.W., Geelen J.L., van der Noordaa J., 1980. Human cytomegalovirus DNA: physical maps for restriction endonucleases *Bgl*II, *Hind*III and *Xba*I. J. Gen. Virol., 49(1): 1–21. doi: 10.1099/0022-1317-49-1-1.

Wilfinger W.W., Mackey K., Chomczynski P., 1997. Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity. Biotechniques, 22: 471-481. PMID: 9067025.

Xia J., Bai C., Wang C., Song X., Huang J., 2015. Complete genome sequence of *Ostreid herpesvirus-1* associated with mortalities of *Scapharca broughtonii* broodstocks. Virol. J., 12: 1-9. doi: 10.1186/s12985-015-0334-0.

Yáñez R., Rodríguez J.M., Nogal M.L., Yuste L., Enríquez C., Rodríguez J.F., Viñuela E., 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology, 208: 249-278. doi:10.1006/viro.1995.1149.

Yoshino T.P., Bickham U., Bayne C.J., 2013. Molluscan cells in culture: primary cell cultures and cell lines. Can. J. Zool., 91(6): 394-404. doi: 10.1139/cjz-2012-0258.

2.3. Multi-site tests: a study of OsHV-1 and disease co-factors in field environment

In oyster farms, as in other aquaculture facilities, diseases can induce a dramatic drop of commercial incomes. Whether a pathogen would cause disease or not, depends not only on the virulence of the pathogen, but also on the immune defences of the infected animal. Both factors are influenced by environmental conditions and stressors, which can imbalance the host-pathogen interactions, causing disease. Thus, disease does not generally require just a suitable host and a pathogen to appear, but also a stressful environment. Environmental stress may be biological, for instance due to high stocking densities, chemical, physical, such as excessive temperature, or procedural due to handling and processing (Mulcahy and Roch, 2009).

In the last decades, mass mortalities have been observed in various marine invertebrates around the word, in both farmed and wild populations, commonly associated to infectious diseases (Mydlarz et *al.*, 2006). In the recent years, even if the most dramatic losses occurred in young Pacific oysters, other commercial species of molluscs suffered mass mortality events in France such as the blue mussel *Mytilus edulis*, the cockle *Cerastoderma edule*, the clam *Ruditapes* sp., the scallops *Pecten maximus* and *Chlamys* sp., and also the abalone *Haliotis tuberculata* (REPAMO, 2014; 2015). The perception is of an ecological crisis in the oceans related with the deterioration of ocean health (Lafferty *et al*., 2004). Global changes in surface seawater temperature have often been suspected to be responsible for the increase in prevalence and severity of disease outbreaks. Temperature is therefore an important triggering factor of disease epidemics. For example, recent mortalities affecting larval and juvenile bivalves, including *C. gigas* in the USA were associated to *V. tubiashii* blooms in the coastal environment, which were in turn linked with the mixing of unusually warm surface seawater and intermittently deep and cool upwelling seawater (Elston *et al*., 2008).

In France, mass mortality events of *Crassostrea gigas* seed, associated with OsHV-1 µVar, have mainly occurred in spring and summer, when the temperature was over 16 °C. The study conducted by Petton *et al*. (2013) clearly showed the influence of seawater temperature on the disease transmission, determining a permissive range between 16.2°C and 21.9°C. In a previous survey conducted in the Mediterranean Thau lagoon, Pernet *et al*. (2012) observed an interruption of the mortality phenomenon when water temperature exceeded 24°C. However, the definition of a temperature threshold seems to be unrealistic. In fact, the permissive range appears to be site dependent as observed in the USA and Australia, where

viral mortalities occurred with higher temperatures (Burge *et al*., 2007; Jenkins *et al*., 2013; Paul-Pont *et al*., 2013b). This induces to suppose that additional factors other than water temperature are involved in the OsHV-1 disease.

Phytoplankton is also a suspected natural vector of viruses in the field. Several experimental studies used the virus–phytoplankton adhesion route to infect primary and secondary consumers (Zhang *et al*., 2006; 2007). Paul-Pont *et al*. (2013a) and Evans *et al*. (2014) sustained the particulate-attached virus theory for OsHV-1.

Moreover, there is circumstantial evidence that certain diseases occur in shellfish at higher prevalence where the environment is degraded and polluted (see review Morley, 2010).

Intrinsic factors of the host may also influence the insurgence of mortalities. Triploid oysters were often preferred to diploids because of their limited gonadal development, faster growth, and better survival. In fact, thanks to a lower reproductive stress due to the limited gonadal development, triploid market-size individuals appeared less sensitive to “summer mortality” (Normand *et al*., 2008; Samain and McCombie, 2008). Otherwise, the ploidy seemed irrelevant in disease associated with OsHV-1 in young individuals (Pernet *et al*., 2010).

Petton *et al*. (2015b) confirmed that the viral load is a predictor of mortality (Oden *et al*., 2011), however, they demonstrated that, in absence of bacteria, a high load of OsHV-1 was not sufficient to induce the full expression of the disease. This study highlights the importance of infectious agents as co-factors in the development of the viral disease.

In general terms, our understanding of the complexity of infections occurring in the natural environment remains limited. Marine systems are extremely different from terrestrial environments, and these differences complicate the application of models developed for terrestrial systems. The aquaculture environment is characterised by a hydrodynamic connectivity between different farming sites that exacerbate the risk of pathogen diffusion (McCallum *et al*., 2004). Farmed animals establish an extremely close relation with their environment, being affected by numerous components, which is arduous or even unattainable to reproduce in laboratory conditions. For these reasons, in order to determine the environmental drivers of infection by OsHV-1 and disease development in young Pacific oysters, we decided to carry out a two years-long multi-sites test, choosing the experimental sites on the basis of their environmental characteristics. We used an integrative approach that took into account environmental and host parameters. The aim was to define predictive

models of oyster mortality and disease transmission to mitigate their impact by the subsequent development of management tools for oyster farming.

2.3.1. Materials and Methods

Study sites

The survey conducted between May 2014 and December 2015 involved nine sites, located along both Adriatic and Tyrrhenian Italian coasts (Figure 29A). For the year 2015, two additional sites in Normandy (France) have been included (Figure 29B).

Fig.29A Study sites in Italy A;B: in France . Red star: 2014 campaign; Yellow star: 2015 campaign; Light-Blue star: 2015 campaign with only two samplings (in June and November).

Fig.29B Study sites in France . Yellow star: 2015 campaign.

The sampling sites were representative of four different types of marine environment (Table 8). Four sites were located within a lagoon: Caleri (Rovigo), Varano (Foggia), Orbetello (Grosseto), and San Teodoro (Olbia-Tempio); three in a gulf: La Spezia, Olbia (Olbia-Tempio) and Gaeta (Latina); two in open waters: Caorle (Venezia), and Giulianova (Teramo); and the two French sites, Meuvaines and Baie des Veys in the intertidal foreshore.

Site	Type of Environment	Latitude	Longitude
Caorle	Open Waters	45°31'37.94"N	12°51'9.00"E
Caleri	Lagoon	45° 5'12.72"N	12°19'37.90"E
Giulianova	Open Waters	42°48'22.11"N	14° 0'0.81"E
Varano	Lagoon	41°52'47.23"N	15°43'26.18"E
Gaeta	Gulf	41°14'4.63"N	13°36'4.47"E
Orbetello	Lagoon	42°25'56.26"N	11° 9'51.30"E
La Spezia	Gulf	44° 4'32.24"N	9°51'58.34"E
Olbia	Gulf	40°55'3.79"N	9°31'47.24"E
San Teodoro	Lagoon	40°48'40.94"N	9°40'28.86"E
Meuvaines	Inter-tidal foreshore	49°21'6.82"N	0°32'39.91"O
Baie des Veys	Inter-tidal foreshore	49°23'22.76"N	1° 5'42.62"O

Table 8 Description of the study sites concerned by the survey campain.

The farming site of Caorle is located in open waters at about 5 km from the coastline (Figure 30). In this area the sea depth is about 15-18 meters with a detrital - sandy seabed. The site is situated between the Livenza and Piave Rivers mouths, which can drain huge volumes of freshwater into the sea during the rainiest months, influencing the salinity of extended areas of marine water. Furthermore, inland waters transport an important quantity of

sediments, which contribute to increase the seawater turbidity, and a multitude of contaminants of industrial, agricultural and geogenic origins.

Fig. 30 "Caorle" study site and main freshwater tributaries.

This production area is mainly devoted to mussel *M. galloprovincialis* farming and in a lesser extent to *C. gigas*. The innovative "New Zealand's rope mussel on-growing system" is used while oysters are reared in lantern-nets hung to a longline. Since the production in this area is organic, the animal density is quite low.

The northern and northwestern part of the Adriatic is characterised by a complex system of transitional environments that includes, amongst others, the Marano, Venezia, and Caleri Lagoons. In Caleri, as in other parts of the northern lagoons, the water temperature is high and the oxygen concentration low in summer, partly due to the shallowness of the water. However, respect to other coastal lagoons, this area is mostly influenced by open sea environmental conditions because there is no direct entrance of river water and good exchanges with the open sea. Moreover, the study site is located near the 200 meter wide channel that connects the lagoon to the sea (Figure 31). The clam *Ruditapes* sp. farming is intensively performed in the entire lagoon. Even if only experimental trials of oyster culture have been conducted in this site, a conspicuous presence of wild *C. gigas* is present.

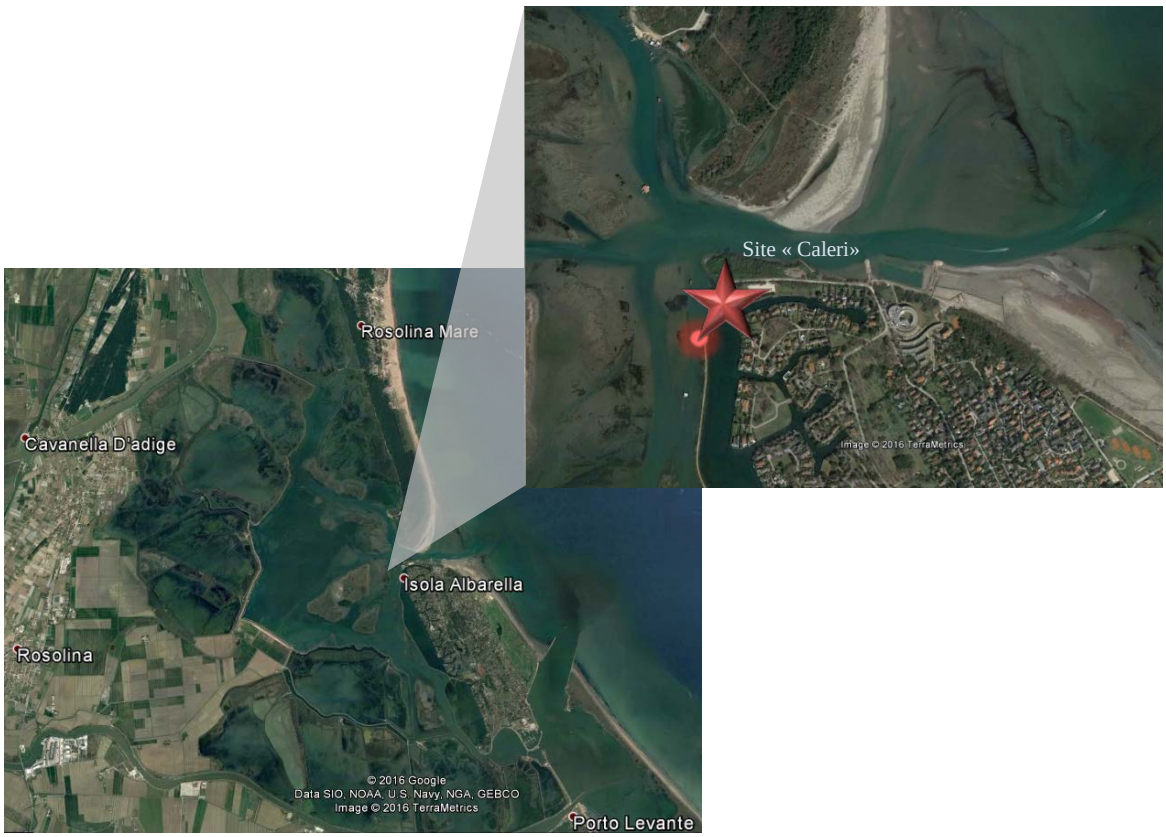

Fig. 31 "Caleri" study site.

The site of Giulianova, along the central Adriatic coast, is located in open-waters, 5 km from the coastline (Figure 32), and it is dedicated to *M. galloprocincialis* farming on longlines. Being situated further South than the previously described locations, it receives a lower influence of freshwater income from the Northern large rivers.

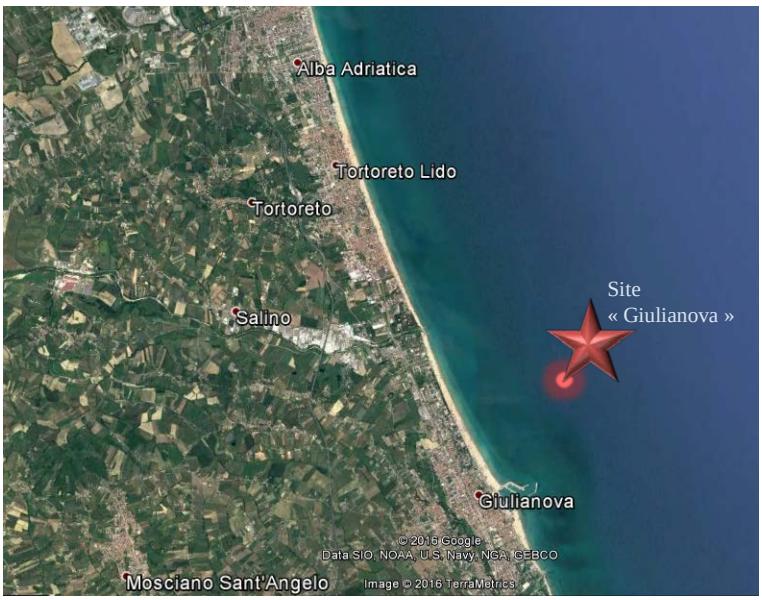

Fig. 32 "Giulianova" study site.

Varano Lake is the most southerly Adriatic site. It is located in the northern part of Gargano National Park and weakly communicates with the open sea via two artificial canals. The survey was conducted in the almost central zone of the lake, where the maximum depth is about 6 m (Figure 33). The area has poor industrialization; the basin receives wastewaters from the surrounding villages and agricultural drainage water. The salinity of the surface layer, where the Pacific oysters were collected, is lower than 30‰ and stable all year round because of the presence of underwater springs (Spagnoli *et al.*, 2002). Different mollusc species are produced: clams, mussels and flat oysters *C. gigas.* However, in summer, the high water temperatures and the development of a lush vegetation of macrophytes on the lake bottom, induce periodic but severe conditions of hypoxia, compelling the farmers to transfer their stocks of mussels and flat oysters to open water facilities.

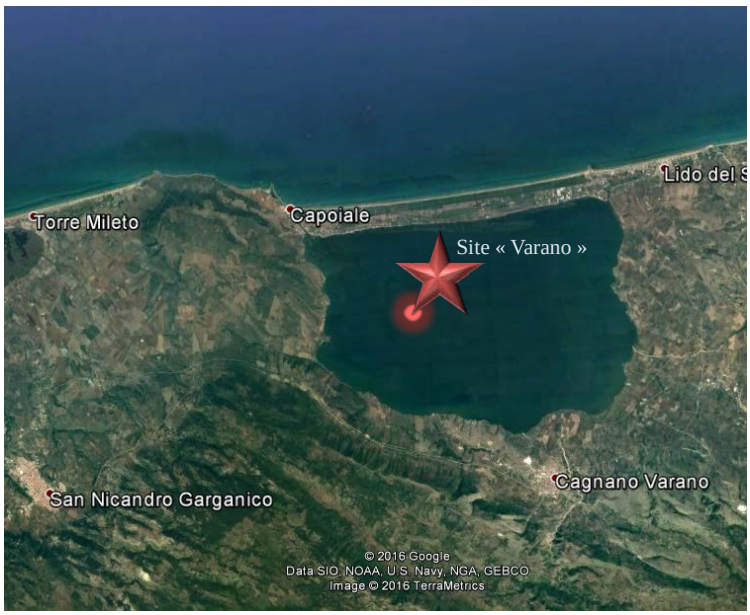

Fig. 33 "Varano" study site.

The next five stations concerned by the study were located in the Tyrrhenian Sea. If compared to Adriatic, it is characterised by oligotrophic conditions and a relative higher and stable salinity.

In Gaeta, the numerous facilities for mussel and finfish culture occupy a portion of the large open gulf (Figure 34). To date, no oyster production is carried out even if some trials have been performed in previous years. Mussels are intensively farmed in socks attached to ropes, which are shorter longlines used in open waters and sustained by buoys. Near the mussel farms, about hundred floating cages are used for sea bream and sea bass farming.

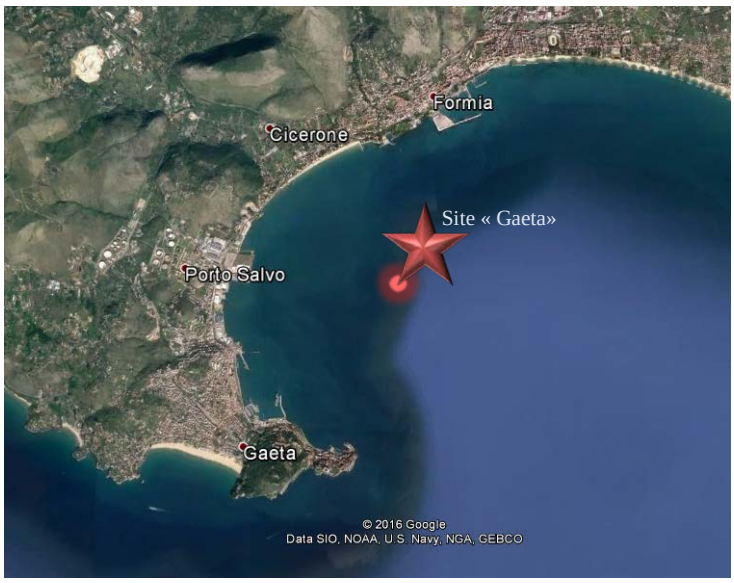

Fig.34 "Gaeta" study site.

The Orbetello Lagoon is an area of high environmental interest and a designated International Relevant Wetland site. Aquaculture activities are conducted, mainly represented by sea bass production. Oyster farming has been practiced for few years but this activity is stopped nowadays. The lagoon is split into two basins that are connected together and the mean water depth about 1 m; the Northern basin communicates with the open sea via two canals, Nassa and Fibbia. The sources of anthropogenic contamination are now represented by agriculture wastewaters mainly, but until 1991, a chemical factory manufacturing granular fertilizers released waste metals into the western part of the basin. The survey was carried out near the entrance of the Nassa Canal in the lagoon (Figure 35).

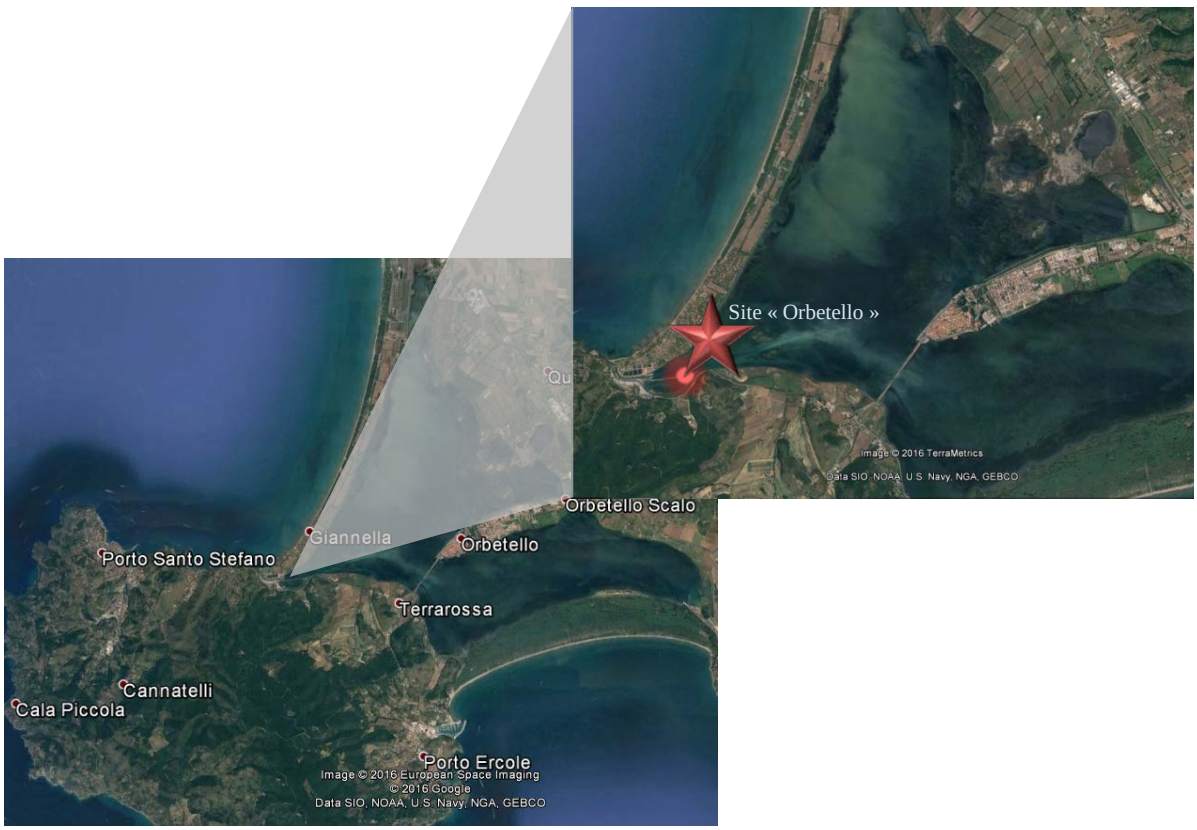

Fig. 35 "Orbetello" study site.

The La Spezia Gulf is a quite enclosed bay, where the mollusc aquacultural production of mussels and Pacific oysters is carried out along the backside of the dam erected to protect the central zone of the gulf from coastal storms (Figure 36). Mussels are farmed in short socks while lantern-nets, while "Australian baskets" are used for oyster cultivation. The mollusc density in the producing area is high.

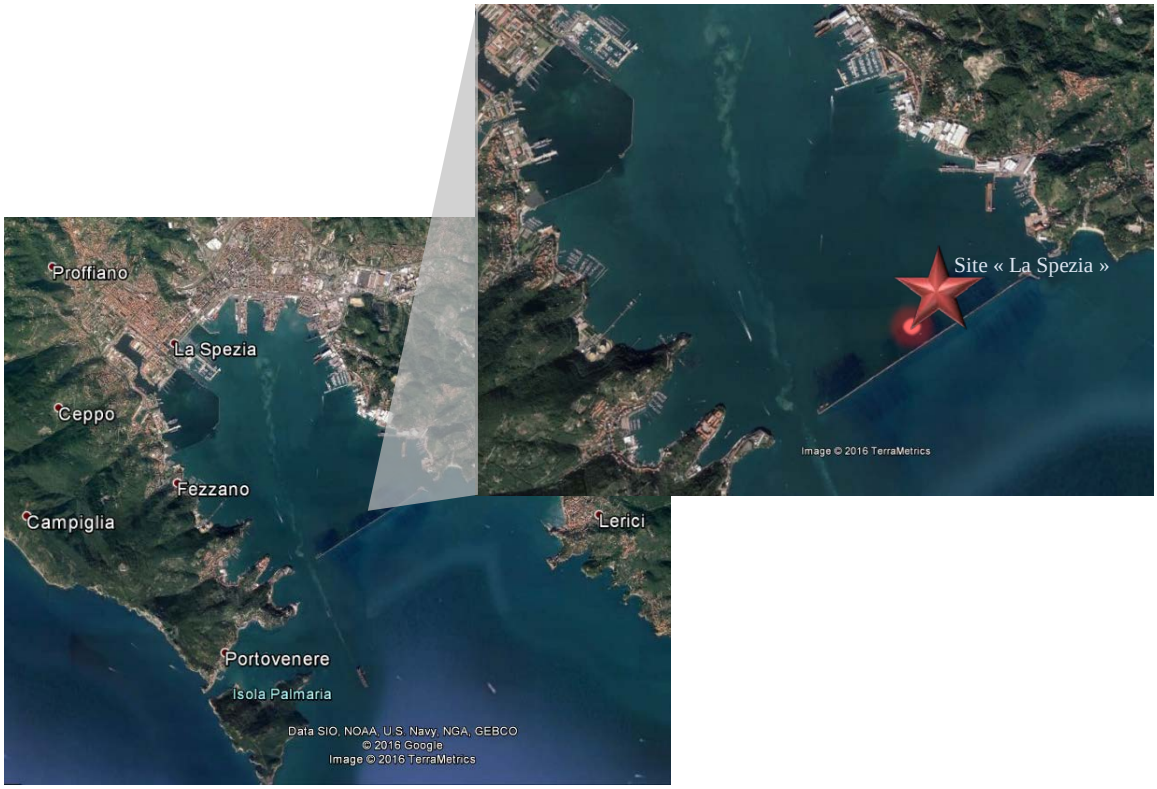

Fig. 36 "La Spezia" study site.

The last two Italian sites have been chosen in Sardinia and are quite close one to each other. Nevertheless, they are characterised by different environmental conditions. Olbia is a very enclosed gulf, where intensive mussel production is conducted (Figure 37).

Fig. 37 "Olbia" study site

On the contrary, San Teodoro lagoon is a shallow coastal pond (Figure 38) connected to the sea through a narrow mouth and characterised by sandy bottoms and an average depth of 0.7 meters. It receives nutrient rich freshwater from the Rio San Teodoro and Rio Filicani. The basin is scarcely exploited for traditional fishing, but it represents the most important Italian site for Pacific oyster production. During the farming cycle, pearl-nets, Australian baskets and oyster floating bags are used.

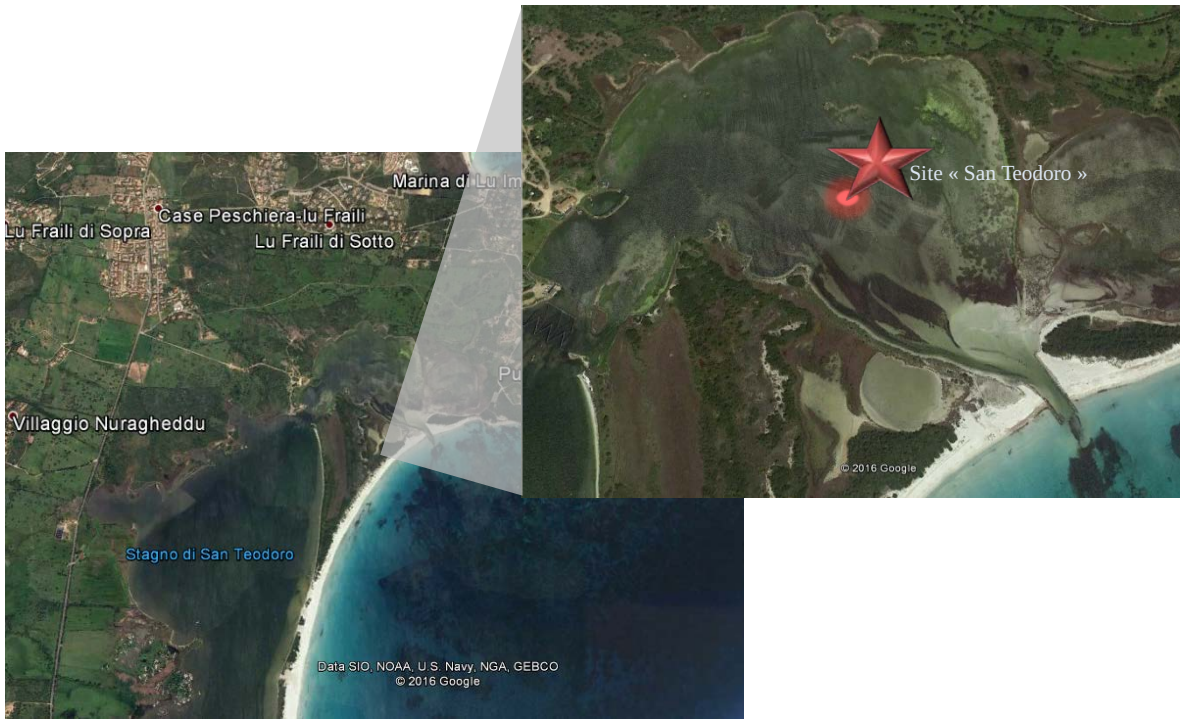

Fig. 38 "San Teodoro" study site.

In France oyster farming is performed in the intertidal zone, in areas characterised by a slight declivity and a sandy sea floor. The two French sites are located in Normandy along the Southern Channel coast. The Baie des Veys is a highly productive area for oyster farming. In fact it is situated in a large estuary where four important rivers (Douve, Taute, Vire, and Aure Rivers) discharge their waters, enriched in nutrients (Figure 39). These freshwater supply influences also the salinity of the surrounding environment and the possible presence of pollutants.

The environmental conditions of the site of Meuvaines are very different with respect to Baie des Veys, being far from important rivers (Figure 40). This area is also mostly exposed to a very powerful hydrodynamics.

Fig.39 “Baie des Veys” study site

Fig. 40 “Meuvaines” study site.

Oyster allocation in experimental sites

In each site previously described and at different times between 2014 and 2015, we allocated Pacific oyster spat originated from a French hatchery. For each allocation date and ploidy, all individuals belonged to the same original batch.

- *allocation campaigns for the year 2014*

The first campaign was conducted at the end of May 2014. At their arrival the two batches of spat, triploid and diploid consisting in 14,000 individuals each, have been divided in 54 batches of about 250 animals. The individual length was defined as T5, which means they were retained by a sieve opening of maximum 5 mm, and their average weight was 87 mg.

The remaining 500 individuals were kept for successive analyses. In each site, except for San Teodoro, we placed two lantern-nets containing 1,500 young oysters each, distributed as illustrated in Figure 41. All the lantern-nets were clearly identified and securely fastened to the horizontal rope.

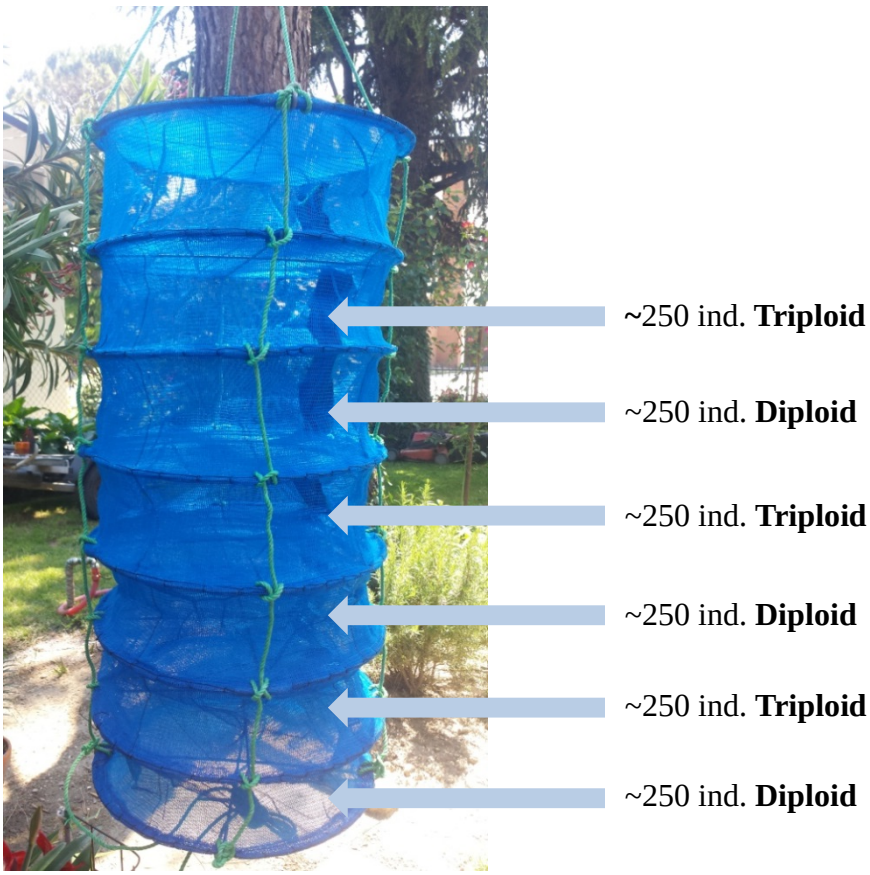

Fig. 41 Distribution of young oyster individuals in the lantern-net

In San Teodoro, the lagoon was not deep enough to place the lantern-nets. Thus, floating oyster bags were used. In fact, this technique is the conventional method employed for oyster farming in this location (Figure 42). Seven hundred and fifty individuals were inserted in each bag, resulting in two bags with diploid specimens and two with triploids.

Fig. 42 Floating oyster bags in the San Teodoro Lagoon.

The second allocation campaign was carried out at middle July 2014, using the same operating procedure.

Finally, a third and scanter campaign was conducted at the end of September 2014: only 250 individuals T5 triploid were placed in the second floor of a lantern-net in each sites except for San Teodoro where an additional floating bag was used. In fact, the hatchery was unable to provide diploid individuals in those days.

Spat was placed firstly in lanterns with a 2.5 mm-meshed net (Figure 43A). During the course of the experimentation, on the basis of the different growth rates observed in the various sites, oyster batches were moved in new lanterns with a 12 mm-mesh net (Figure 43B) as soon as their minor dimension was bigger than the opening of the mesh. In the same way, oysters kept in 4 mm-meshed floating bags at the beginning of the survey were then transferred in new bags with a larger mesh during their growth.

For each site, simultaneously with the last sampling campaign occurred in October 2014 and further described in the next chapter, all the individuals resulting from the three allocations were pooled maintaining diploid and triploid individuals separated and then subdivided in two lanterns with a 14 mm mesh and 10 compartments. The maximum density was 80 individuals per compartment. The same protocol was used in San Teodoro, where lanterns were replaced by floating bags.

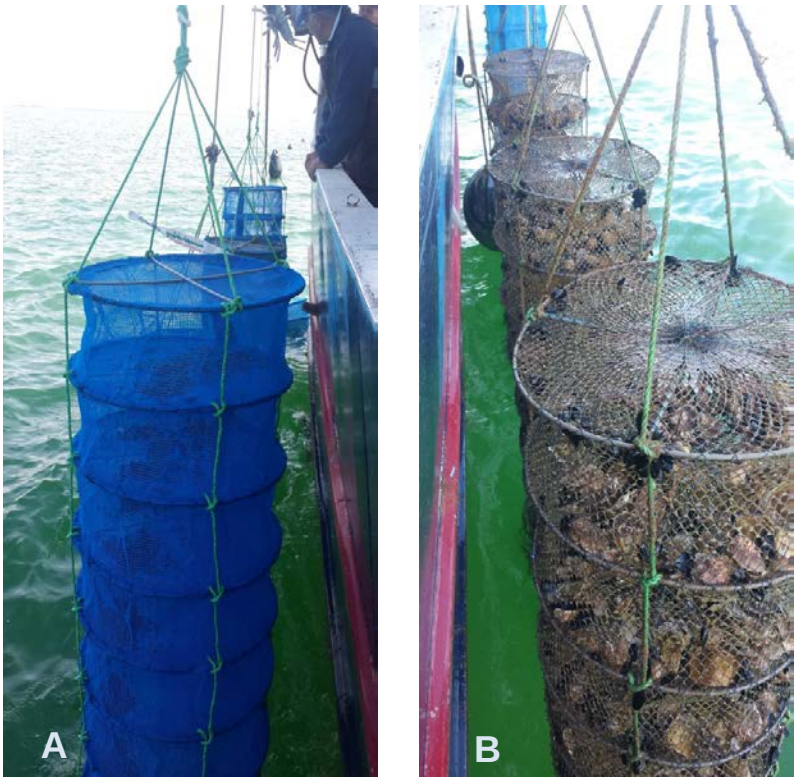

Fig. 43 Different lantern-nets used during the survey. A: 2.5 mm-meshed net; B: 12 mm-meshed net.

- *allocation campaigns for the year 2015*

During 2015, only two allocation campaigns were carried out, involving 30,000 (15,000 diploid and 15,000 triploid) and 7,500 (only triploids) individuals respectively. At their arrival, 500 supernumerary individuals of the three batches were kept for successive analyses.

The first step, in middle April, concerned eight Italian sites (Olbia was excluded to the survey for the year 2015, because of the unwillingness of the farmer) and the two French sites. As in San Teodoro, four oyster bags were used in each French sites with the difference that they were firmly attached and supported by racks, securely set in the sediment and placed in the foreshore area of the sandy coast (Figure 44). The spat allocation protocol was the same as that used in 2014. However, if the size of triploid individuals was comparable to 2014 (T5 with mean weight of 90 mg), diploids were smaller (T2, mean weight 8 mg) and it was impossible to start using the 2.5 mm-meshed lantern directly or the 4 mm-meshed oyster bags. Thus, we solved the problem by inserting these specimens in a 0.8 mm-meshed bag firstly (Figure 45), placed in the compartment of the lantern-net and maintained flattened by anchoring each vertex to the lantern. In France, since the farming devices are under high strain due to repeated bad marine conditions, T2 individual were placed in tubes specially

made (Figure 46). During the following month, the individual size was then sufficient to remove the meshed bag in all Italian sites (Figure 47). In France, two months were necessary instead.

For the second campaign, conducted in middle September, organisational problems have prevented to involve the sites of Giulianova, Varano, Gaeta, Meuvaines and Baie des Veys. In the five other sites 1,500 triploid individuals were placed in the usual nets and, as for other batches, further 500 specimens were kept for successive analyses.

Fig. 44 Technique used in the sites of Meuvaines and Baie des Veys for oyster farming in the intertidal zone. (photo S. Trancart)

Fig. 45 Meshed-bags placed in the lantern-net and used to contain diploid individuals during the first month

Fig. 46 Device used for the first months in France for the T2 diploid individuals.

Fig. 47 Diploid individuals after one month in the meshed-bad and ready to be transferred in the lantern-net directly.

Sampling campaigns

The planned sampling frequency was every 21 days for each site and for the entire duration of the survey. In some cases it was not possible to reach the experimental site exactly in the pre-established date because of bad weather, vessel breakdown, or insufficient tide coefficient, the last reason applying only for French sites. However, at least one sampling by month was carried out in each site (for sampling dates refer to Appendix A2-A3). The survey was performed between May and October 2014 and between April and December 2015. Only for the site of San Teodoro, some samples were collected during the period between the two annual surveys, and in January and February 2016.

- *mortality recording*

During each sampling and in each experimental unit, empty shells were counted to determine the mortality rate between two successive samplings and for both ploidies. Dead oysters were removed and moribund specimens were collected and packed separately to be analysed.

- *sampling for virological and bacteriological analyses*

Since April 2014, periodical sampling consisted in the random collection of five specimens per each compartment of the two lantern-nets, or of 15 individuals per each oyster bag, leading to the sampling of 30 triploid and 30 diploid individuals. The two pools of oysters were conserved separately, immediately placed in a refrigerated box and processed

within 24-48 hours. Then, since July/August, the same protocol was also applied to oysters of the second allocation (July 2014), resulting in the simultaneous collection of four pools of 30 individuals. Finally, after the last deployment of spat in September, an additional pool of 30 triploid individuals was added to the collection.

For the year 2015, a similar sampling protocol was applied with the difference that in addition to the collection of spat deployed in the various sites in 2015, 30 triploid and 30 diploid individuals placed *in loco* in 2014 were sampled at once. Unfortunately, a huge storm has destructed the farming site of Giulianova in March 2014 and all the lantern-nets have been lost, while in Varano oysters placed in 2014 have not been conserved by the farmer. The mortalities suffered by the oysters stocks in Caleri in 2014 have compelled us to perform only two sampling of individuals allocated in 2014. The sites of Giulianova and Varano have been sampled only two times in 2015, and the site of Gaeta three times.

A summary of the sampling operations is reported in Tables 9 and 10.

site	number	ploidy	allocation date	sampling dates	site	number	ploidy	allocation date	sampling dates
	30	triploid	May 2014	19/06/14; 07/07/14		30	triploid	May 2014	26/06/14; 09/07/14
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	May 2014			30	triploid	May 2014	
	30	diploid	May 2014	21/07/14; 06/08/14; 02/09/14; 30/09/14		30	diploid	May 2014	30/07/14; 28/08/14; 25/09/14; 08/10/14
	30	triploid	July 2014			30	triploid	July 2014	
Caorle	30	diploid	July 2014		Orbetello	30	diploid	July 2014	
	30	triploid	May 2014			30	triploid	May 2014	
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	July 2014	18/10/14		30	triploid	July 2014	23/10/14
	30	diploid	July 2014			30	diploid	July 2014	
	30	triploid	September 2014			30	triploid	September 2014	
	30	triploid	May 2014	21/06/14; 07/07/14		30	triploid	May 2014	26/06/14; 09/07/14
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	May 2014			30	triploid	May 2014	
	30	diploid	May 2014	21/07/14; 06/08/14; 02/09/14; 02/10/14		30	diploid	May 2014	30/07/14; 28/08/14; 25/09/14; 08/10/14
	30	triploid	July 2014			30	triploid	July 2014	
Caleri	30	diploid	July 2014		La Spezia	30	diploid	July 2014	
	30	triploid	May 2014			30	triploid	May 2014	
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	July 2014	16/10/2014; 30/10/14		30	triploid	July 2014	23/10/14
	30	diploid	July 2014			30	diploid	July 2014	
	30	triploid	September 2014			30	triploid	September 2014	
	30	triploid	May 2014	20/06/14; 04/07/14; 24/07/14		30	triploid	May 2014	07/07/14
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	May 2014			30	triploid	May 2014	
	30	diploid	May 2014	07/08/14; 17/09/14; 01/10/14		30	diploid	May 2014	31/07/14; 03/09/14; 01/10/14
	30	triploid	July 2014			30	triploid	July 2014	
Giulianova	30	diploid	July 2014		Olbia	30	diploid	July 2014	
	30	triploid	May 2014			30	triploid	May 2014	
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	July 2014	17/10/14		30	triploid	July 2014	23/10/14
	30	diploid	July 2014			30	diploid	July 2014	
	30	triploid	September 2014			30	triploid	September 2014	

Table 9 Description of the sampling campaigns conducted in 2014 with the details of sample composition per date of sampling

site	number	ploidy	allocation date	sampling dates	site	number	ploidy	allocation date	sampling dates
Varano	30	triploid	May 2014	20/06/14; 04/07/14; 23/07/14	San Teodoro	30	triploid	May 2014	07/07/14
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	May 2014	07/08/14; 03/09/14; 01/10/14		30	triploid	May 2014	31/07/14; 03/09/14; 01/10/14
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	July 2014			30	triploid	July 2014	
	30	diploid	July 2014			30	diploid	July 2014	
	30	triploid	May 2014	17/10/14		30	triploid	May 2014	23/10/14
	30	diploid	May 2014			30	diploid	May 2014	
	30	triploid	July 2014			30	triploid	July 2014	
	30	diploid	July 2014			30	diploid	July 2014	
	30	triploid	September 2014			30	triploid	September 2014	
Gaeta	30	triploid	May 2014	27/06/14; 10/07/14					
	30	diploid	May 2014						
	30	triploid	May 2014	31/07/14; 28/08/14; 25/09/14; 08/10/14					
	30	diploid	May 2014						
	30	triploid	July 2014						
	30	diploid	July 2014						
	30	triploid	May 2014	23/10/14					
	30	diploid	May 2014						
	30	triploid	July 2014						
	30	diploid	July 2014						
	30	triploid	September 2014						

Table 9bis Description of the sampling campaigns conducted in 2014 with the details of sample composition per date of sampling.

site	number	ploidy	allocation date	sampling dates
Caorle	30	triploid	2014	14/04/15
	30	diploid	2014	
	30	triploid	2014	18/05/15; 09/06/15; 08/07/15; 30/07/15; 17/08/15; 04/09/15; 16/09/14; 07/10/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	2014	18/11/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	September 2015	
Caleri	30	triploid	2014	14/04/15
	30	diploid	2014	
	30	triploid	May 2014	18/05/15
	30	diploid	May 2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	April 2015	11/06/15; 08/07/15; 30/07/15; 17/08/15; 04/09/15; 16/09/15
	30	diploid	April 2015	
	30	triploid	April 2015	06/10/2015; 18/11/15
	30	diploid	April 2015	
	30	triploid	September 2015	
Giulianova	30	triploid	April 2015	10/06/15; 29/10/15
	30	diploid	April 2015	
Varano	30	triploid	April 2015	10/06/15; 29/10/15
	30	diploid	April 2015	
Gaeta	30	triploid	2014	16/04/15
	30	diploid	2014	
	30	triploid	2014	25/06/15; 05/12/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	

Table 10 Description of the sampling campaigns conducted in 2015 with the details of sample composition per date of sampling.

site	number	ploidy	allocation date	sampling dates
Orbetello	30	triploid	2014	16/04/15
	30	diploid	2014	
	30	triploid	2014	26/05/15; 24/06/15; 15/07/15; 29/07/15; 12/08/15; 03/0915; 16/09/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	2014	14/10/15; 05/12/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	September 2015	
La Spezia	30	triploid	2014	16/04/15
	30	diploid	2014	
	30	triploid	2014	26/05/15; 24/06/15; 15/07/15; 12/08/15; 03/09/15; 16/09/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	2014	15/10/15; 04/12/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	September 2015	

Table 10bis Description of the sampling campaigns conducted in 2015 with the details of sample composition per date of sampling.

site	number	ploidy	allocation date	sampling dates
San Teodoro	18	triploid	2014	21/01/15
	12	triploid	2014	11/03/15
	3	triploid	2014	20/03/15
	15	triploid	2014	27/03/15
	7	triploid	2014	31/03/15
	6	triploid	2014	03/04/15
	30	triploid	2014	07/04/15
	30	diploid	2014	
	30	triploid	2014	25/05/15; 15/06/15; 15/07/15; 05/08/15; 03/09/15; 16/09/15; 28/09/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	2014	26/10/2015; 18/11/15
	30	diploid	2014	
	30	triploid	April 2015	
	30	diploid	April 2015	
	30	triploid	September 2015	
	13	triploid	Maggio 2014	16/12/15
	5	triploid	Aprile 2015	
	24	triploid	Maggio 2014	19/01/16
	12	triploid	Aprile 2015	
	7	triploid	Maggio 2014	15/02/16
	18	triploid	Aprile 2015	
Meuvaines	30	triploid	Avril 2015	03/06/2015; 01/07/2015; 03/08/2015; 15/09/15
	30	diploid	Avril 2015	
Baie des Veys	30	triploid	Avril 2015	11/06/2015; 02/07/2015; 03/08/15; 16/09/2015
	30	diploid	Avril 2015	

Table 10ter Description of the sampling campaigns conducted in 2015 with the details of sample composition per date of sampling.

Biometry

During the entire survey conducted in 2014, the growth of the oysters allocated in May was monitored. At their arrival at the laboratory, biometric measures were carried out on all the sampled specimens. Shell length, defined as the greatest anteroposterior length, was measured with a manual calliper.

Prevalence and DNA quantification of OsHV-1 and *V. aestuarianus* in oyster flesh

For all the sampled batches, each consisting in 30 individuals, pools of the minced tissues of five individuals were prepared and DNA was extracted on 50±0.5 mg of pooled tissues, according to the protocol illustrated in Appendix B.1. The quantitative real-time PCR method, used for the detection and quantification of OsHV-1, is detailed in Appendix B.2. It included an Internal Control IC to exclude false negative results imputable to the presence of PCR inhibitors. The assay validity was evaluated as reported in Appendix B.2. The pools tested positive were then analysed individually, repeating the same protocol.

A real-time PCR assay, based on TaqMan® technology (Applied Biosystems) was also carried out for the detection and estimation of the amount of *V. aestuarianus* DNA copies in the extract. The diagnostic test is based on the amplification of part of the dnaJ gene, encoding heat shock protein 40 (Saulnier *et al*., 2009). We used the primer pair dnaJ- F (5' GTATGAAATTTTAACTGACCCACAA 3') and dnaJ-R (5' CAATTTCTTTCGAACAACCAC 3') with the dnaJ-probe (5' TGGTAGCGCAGACTTCGGCGAC 3'). The reaction volume of 25 µL contained 12.5 µL of Takara Premix Ex TaqTM 2X (Takara Bio Inc., Shiga, Japan), 0.5 µL of each primer (20 µM), 0.5 µL of probe (10 µM), 9 µL of DNA/nuclease-free water, and 2 µL of extracted DNA (replaced by 2 µL of DNA/nuclease-free water as negative control). Standard curves were prepared according to the EURL for Mollusc Diseases standard operating procedure (http://www.eurl-mollusc.eu/content/download/90299/1109300/version/1/file/Vaestuarianus+_RealTimePCR_editionN%C2%B03.pdf) using dilutions of bacterial DNA suspension. We used the same thermal cycling conditions as for OsHV-1 detection. The result was expressed in copies of *V. aestuarianus* DNA (GU)/50 mg of oyster tissue. The assay was considered valid only if the extraction and the negative controls did not present any amplification, and if the regression coefficient of the standard curve was at least 0.98 and the slope between -4.115 and -2.839. A sample was considered positive only if the Ct value was ≤38.5. A sample was considered a true negative only if amplification of the IC was successful.

Variant characterization

To define which OsHV-1 genotype(s) infected the samples tested positive with real-time PCR we applied the same protocol used in Publication 1 and reported in Appendix B.3. It is based on the sequencing of three regions of the viral genome: C region, ORFs 42/43, and ORFs 35-38.

Bacteriological analysis

Several *V. splendidus* strains and some related species have been frequently recognised as pathogenic for bivalve molluscs. *C. gigas* was frequently affected by these vibrios (Sugumar *et al*., 1998; Lacoste *et al*., 2001; Le Roux *et al*., 2002; Garnier *et al*., 2007). Thus, in 2014, when moribund individuals were found during samplings and their size was sufficient for haemolymph draw, bacteriological analyses were carried out with the aim to evidence or exclude the presence of these species. Haemolymph was drawn from the adductor muscle or pericardial cavity of 15 moribund individuals (six from Caleri and three from Caorle, Varano, and San Teodoro) using a 1 mL needle. Forty µL of haemolymph and 40 µL of the 10^{-1} dilution in Zobell broth were spread directly on Zobell agar plates and incubated for 48 h at 22 °C. An estimation of the bacterial load was done. All the colonies characterised by a peculiar aspect were reisolated in pure culture on Zobell agar incubated for 48 h at 22 °C, in order to collect at least one colony by morphology type. When a low variability was observed on a sample plate, at least twelve colonies were collected anyway. Identification of strains was performed through biochemical tests, and in particular for vibrios, using a dichotomous key developed on the basis of information collected in literature (Noguerola and Blanch, 2008). All these strains were also subjected to MALDI-TOF analysis, performed in duplicate, after 48 h of growth on Zobell agar at 22°C.

However, since vibrios from the Splendidus clade are closely related, a potent tool was needed to obtain a correct identification of them. Thus, we developed a Multi Locus Sequence Analysis (MLSA), specific to *V. splendidus* group, based on fragments of 5 housekeeping genes: *atpA*, *ftsZ*, *mreB*, *rpoD* and *topA*, as reported in **Publication 3**.

Finally, since no information was available on the haemolymph microflora outside mortality events, a new protocol was followed in 2015 and was applied to individuals sampled in San Teodoro, La Spezia, Caorle and Caleri. Thus, in early July, early September and late October 2015, haemolymph was drawn from the adductor muscle or pericardial cavity of three apparently healthy individuals per ploidy as explained above. The same performance was carried out in moribund individuals if a mortality event was in course during sampling. A total

count of the grown colonies was performed for each sample in order to compare the bacteria loads. As it was not possible, for reasons of time, to apply the method exposed in **Publication 3** to the strains isolated from oysters in 2015, the totality of the strains has been then stocked at -80°C in a solution composed of 60% of the 24 hours-bacterial culture in Zobell broth and 30% of glycerol to be analysed in future.

Histology

Conventional histological examination was carried out only in moribund specimens collected during sampling.

Publication 3:

Aquaculture 464 (2016) 420–427

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Multilocus sequence analysis of *Vibrio splendidus* related-strains isolated from blue mussel *Mytilus* sp. during mortality events

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ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form 12 July 2016
Accepted 19 July 2016
Available online 21 July 2016

Keywords:
Multilocus sequence analysis
Mytilus sp.
Vibrio splendidus-related strains
Mortality

ABSTRACT

One of the most widely European farmed mollusc, the mussel *Mytilus* sp., has been subjected to massive mortalities located in Charente-Maritime (France) in spring 2014. The national surveillance network for mollusc health has reported a systematic detection of *V. splendidus* in all dying batches. *V. splendidus* is the type species of a clade composed of almost 20 known strains with variable pathogenicity on bivalves. In our study, we first developed a Multi Locus Sequence Analysis (MLSA), specific to *V. splendidus* group, based on fragments of 5 housekeeping genes: *atpA*, *ftsZ*, *mreB*, *rpoD* and *topA*. This tool was validated on reference strains and compared with individual gene analyses. It allowed a useful and reliable classification of *V. splendidus* closely-related strains. Thanks to MLSA, we then tried to classify genetically 23 strains isolated from healthy or dying mussels. 21 were classified within the Splendidus clade: in *splendidus* cluster (38%), in *tasmaniensis* cluster (24%), in *artabrorum* cluster (24%) and on distinct branches (14%). All of them were tested by injection to healthy adult mussels to identify possible pathogenic strains. Experimental trials revealed the presence of a strain called M3H, allied with the *splendidus* cluster and able to induce mortality in mussels with rates up to 80%. The M3H virulence was demonstrated by the recovery of the injected strain in dying animals, resulting from repeated experimental infections. Further work should be now conducted to explore the pathogenicity of the M3H strain towards different mussel batches and under various conditions.

Statement of relevance: Our MLSA is useful to identify related *V. splendidus* strains

1. Introduction

The mussel *Mytilus* sp. has become one of the major farmed mollusc species in France which ranks third in Europe, with a production of 73,000 tons per year. Two species are cultivated along French coasts: *Mytilus edulis*, also known as blue mussel and *Mytilus galloprovincialis* called Mediterranean mussel. Natural hybrids of the two species are commonly found in the environment. Mussel culture can either be on wooden stakes called "bouchots" or in ropes, mostly in the Mediterranean, with a 15-month production cycle. Contrary to other bivalve cultures, mussel farming did not suffer many massive mortalities in the last decades. Only a few studies reported these kinds of events, generally ascribed to changes in environmental conditions (Myrand et al., 2000; Peperzak and Poelman, 2008; Tsuchiya, 1983). In spring 2014, massive mussel mortalities have been reported in Charente-Maritime with losses reaching up to 80%. Furthermore, a survey performed by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) through the REPAMO network (REseau de PAthologies de Mollusques), has recorded 12 mortality episodes, in different mussel batches along the French coast, during the first half of 2014 and concerned all ages. Several mussel pathogens frequently found in bivalves, including the OIE-listed *Marteilia refringens*, have been looked for but *Vibrio splendidus* was the only microorganism detected in all samples (Bechemin et al., 2014; Travers et al., 2014). Several *V. splendidus* strains and some related species have been frequently recognized as pathogenic for mollusc bivalves. The Pacific oyster *Crassostrea gigas* was frequently affected by these vibrios (Garnier et al., 2007; Lacoste et al., 2001; Le Roux et al., 2002; Sugumar et al., 1998), but also the clam *Ruditapes decussatus* (Gomez-Leon et al., 2005) and the scallop *Patinopecten yessoensis* (Liu et al., 2013). However, *Vibrio* species are abundantly found in aquatic habitats without any pathogenic significance (Urakawa et al., 1999) and many studies have reported the predominance of *V. splendidus*-related species in healthy bivalve tissues all year long (Beaz-Hidalgo et al., 2010a; Pujalte et al., 2010; Wendling et al., 2014). Thus, these species appear to be part of regular components of farmed aquatic animal microflora or can be accumulated through bivalves filter-feeding habits (Prieur et al., 1990). That is why attention

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http://dx.doi.org/10.1016/j.aquaculture.2016.07.024
0044-8486/

has to be paid to correlate the detection of *V. splendidus*-related species and mortality events.

To date, *V. splendidus* and the related species form a clade composed of at least twelve species, associated or not with mollusc diseases: *V. splendidus*, *V. tasmaniensis*, *V. lentus*, *V. crassostreae*, *V. gigantis*, *V. kanaloae*, *V. chagasii*, *V. fortis*, *V. pomeroyi*, *V. cyclitrophicus*, *V. pelagius*, *V. toranzoniae* (Faury, 2004; Hedlund and Staley, 2001; Le Roux et al., 2002; Macián et al., 2001; Romalde et al., 2014; Thompson, 2003a, 2003b) and five less consensual species: *V. artabrorum*, *V. atlanticus*, *V. celticus*, *V. gallaecicus* and *V. hemicentroti* (Beaz-Hidalgo et al., 2010b, 2009; Dieguez et al., 2011; Kim et al., 2013; Lasa et al., 2013). Classification of *Vibrio* strains, within clades, has been previously performed by genetic methods like Multi Locus Sequence Analysis, generally called MLSA (Sawabe et al., 2013; Thompson et al., 2005). This method is based on the sequencing of multiple housekeeping genes and the construction of concatenated sequences to realize phylogenetic trees. MLSA is thus a reliable tool both to distinguish very closely-related species and define new strains (Gevers et al., 2005; Stackebrandt et al., 2002).

Many previous studies have been conducted to increase knowledge about phylogeny and pathogenicity of Splendidus clade members, isolated during oyster mortality events (Gay et al., 2004a; Nasfi et al., 2015). However, up to now, no report has been published regarding characterization and classification of strains belonging to this clade during and also outside mussel mortalities.

To our knowledge, only few experimental infections, with Splendidus clade strains were carried out on mussels (Ben Cheikh et al., 2016) whereas other bivalves were commonly challenged with, by injection of bacterial suspension into the adductor muscle (De Decker and Saulnier, 2011; Gay et al., 2004b; Liu et al., 2013; Saulnier et al., 2010).

In this paper, *V. splendidus*-related strains were isolated from healthy and dying mussels, in order to be characterized by a Multi Locus Sequence Analysis (MLSA). The aim of the present study was thus to prove that this genetic tool is useful and reliable to identify closely genetic-related species among the Splendidus clade and to classify genetically the strains isolated from both dying and healthy mussels. Finally, the field strain pathogenicity was evaluated through experimental infection trials.

2. Materials and methods

2.1. Reference bacterial strains for MLSA development

Forty-four different reference bacterial strains from the Splendidus clade were used to develop the MLSA tool. Three strains were purchased from national collection (Institut Pasteur, Paris, France): *V. cyclitrophicus* CIP106644T, *V. gigantis* CIP108655 and *V. lentus* CIP107166T; ten reference *V. splendidus* related strains were kindly provided from the Laboratory of Genetics and Pathology (IFREMER, La Tremblade, France): *V. crassostreae* LGP7T, *V. crassostreae* LMG22241, *V. crassostreae* LMG22248, *V. crassostreae* LMG22249, *V. gigantis* LGP13T, *V. pelagius* LMG3897T, *V. splendidus* LMG4042T, *V. tasmaniensis* LGP31, *V. tasmaniensis* LGP32, *V. tasmaniensis* LMG20012T. Eleven other strains from an isolate collection were kindly given by the Genomic of *Vibrio* Research Department (CNRS Roscoff, France): *V. crassostreae* J2.1, *V. crassostreae* J2.9 and *V. crassostreae* ZF_223, *V. cyclitrophicus* 286.55.B3, *V. splendidus* FF_139, *V. splendidus* ZS_173, *V. splendidus* ZS_58, *V. splendidus* ZS_181, V. *splendidus* ZS_2, *V. splendidus* 5S_161 and *V. splendidus* 1S_129; ten from German Collection of Microorganisms and Cell Cultures (DSMZ, Leibniz Institute, Braunschweig, Germany): *V. artabrorum* DSM26480, *V. atlanticus* DSM26479, *V. celticus* DSM26172, *V. chagasii* DSM17138, *V. fortis* DSM19133, *V. gallaecicus* DSM23502, *V. kanaloae* DSM17181, *V. pomeroyi* DSM17180, *V. tasmaniensis* DSM17182 and *V. toranzoniae* DSM 28519; ten from the Spanish Type Culture Collection (University of Valencia, Paterna, Spain): *V. artabrorum* CECT8093, *V. artabrorum* CECT8094, *V. artabrorum* CECT8096, *V. atlanticus* CECT8104, *V. atlanticus* CECT8105, *V. celticus* CECT8133, *V. gallaecicus* CECT7372, *V. hemicentroti* CECT8714, *V. splendidus* CECT8433 and *V. toranzoniae* CECT8091. After validation of the MLSA developed during the present study, the tool was applied on field strains from mussels tested positive for Splendidus clade by PCR. Two reference strains of *V. cortegadensis* CECT7227 and CECT8124 (University of Valencia, Paterna, Spain) were added to this study because of the recent classification of these species closely related to *V. splendidus* clade (Lasa et al., 2013).

All strains were cultivated on Zobell agar for a minimum of 48 h at 22 °C.

2.2. Screening and characterization of bacteria isolated from mussels

Two moribund mussel batches were collected from French farmers in Charente-Maritime and in Chausey (Normandy) in June 2014. Animals were considered dying or moribund when their valves remained open even after a mechanical stimulus. A healthy batch was together taken from the Chausey farm. Eight other healthy batches were sampled between June and August 2014, in different sites from Normandy (Table 2). Tissues of five mussels from a same batch were mashed with scalpels and 50 mg of the homogenate was placed in 200 μL of sterile artificial seawater. After a pulse centrifugation to pellet cellular debris, ten-fold dilutions of supernatant were made and 100 μL of 1:100 and 1:10,000 dilution was spread on Zobell agar incubated for 48 h at 22 °C.

For each mussel batch, around ten predominant bacterial colonies were isolated on Zobell agar to be characterized. DNA extraction was performed by heating a colony placed in 250 μL of DNAse-free water for 10 min at 95 °C. After confirming the successful extraction by DNA quantification with NanoDrop™ 2000c Spectrophotometer (Thermo Scientific™, Waltham, MA USA), two consecutive Taqman® real-time PCRs targeting the 16S gene of *Vibrio* spp. (PCR1) and *V. splendidus* related strains (PCR2) (Nasfi et al., 2015) were carried out. Inclusivity and exclusivity of PCR1 were previously tested (unpublished data). Sequences of specific oligonucleotides are given in Table 1. Typical 25 μL reaction mixtures contained 12.5 μL of Premix Ex Taq® 2× Takara® (Lonza, Verviers, Belgium), 9 μL of DNAse free water, 2 μL of DNA template and 0.5 μL of each primers (20 μM) and probe (10 μM). The thermal cycling profile consisted of 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s (PCR1) or 62 °C for 30s (PCR2). Both real-time PCR were performed with Smart Cycler® (Cepheid, USA).

Only the strains resulted positive with both PCRs were analysed by sequencing.

2.3. Sequencing of housekeeping protein-coding genes

Seven housekeeping genes (*atpA*, *ftsZ*, *gapA*, *gyrB*, *mreB*, *rpoD* and *topA*) were firstly chosen for MLSA development, for their omnipresence, length and adequate variability for an informative level of phylogeny (Wertz et al., 2003; Zeigler, 2003). *Ad hoc* primers were designed with the aid of the Eurofins Genomics design tool (Ebersberg, Germany). Sequences are given in Table 1, also for *gyrB*, after observing that some strains could not be amplified with the universal bacterial primer pair (Yamamoto and Harayama, 1995). MLSA was carried out on the reference strains listed in Section 2.1. Conventional PCR mixtures were composed of 25 μL of Premix Ex Taq® 2× Takara® (Lonza, Verviers, Belgium), 1 μL of forward primer (20 μM), 1 μL of reverse primer (20 μM), 21 μL of DNAse free water and 2 μL of DNA template. PCRs were performed using a T100™ Thermal Cycler (Biorad, France). The thermal program was as follows: (i) 10 s at 95 °C; (ii) 45 cycles of 10 s at 95 °C, 30 s at 60 °C for *topA* or 55 °C for the other genes, 40 s at 72 °C and (iii) a final 3 min at 72 °C. PCR products were analysed with QIAxcel® Advanced System (Qiagen, Courtaboeuf, France) and those with the expected size were sent to Eurofins MWG Operon (Ebersberg, Germany)

Table 1
Genes and oligonucleotides used in this study.

Gene	Amplicon size (bp)	Analysed sequence length (bp)	Oligonucleotide sequence (5′ → 3′)
16S rRNA[a]	111	–	GCGTAAAGCGCATGCAGGT 16S1-F AATTCTACCCCCCTCTACAG 16S1-R TCAGATGTGAAAGCCCGGGG 16S1-P
16S rRNA[b]	153	–	ATCATGGCTCAGATTGAACG SpF1 CAATGGTTATCCCCACATC SpR1 CCCATTAACGCACCCGAAGGATTG SpProbe
atpA	751	591	AAAGTTACAGGTACTGGTCGTATT atpA-F GTCACCAGCTTGCGTTTCGA atpA-R
ftsZ	530	345	AGCGTGAGCAGCGTGATCCA ftsZ-F ATACCGCTACCCATCATTGCATG ftsZ-R
gapA	673	550	CGTGTTACAGCTGARCGTAA gapA-F CTTTAGCATCGAATACTGAAGTTTG gapA-R
rpoD	373	289	ATGATGCAAGACGTATTCAAAGARTTC rpoD-F CACGACGAGCTTTCGCTTCA rpoD-R
gyrB	754	605	ATCACTTCGAATACGAAGGCG gyrB-F GAAGACAGCATTTTATCGAAACG gyrB-R
topA	468	335	ACGTTTTATGGACCGTGTT 3′ topA-F GAGTCAGTACGCATATAAG 3′ topA-R
mreB	472	380	CTGGTGCTCGYGAGGTTTAC mreB-F CCRTTYTCTGAKATATCAGAAGC 3′ mreB-R

[a] Taqman® Real-Time PCR specific to *Vibrio* genus.
[b] Taqman® Real-Time PCR specific to *Vibrio splendidus*-related strains.

to be purified and subsequently sequenced on both strands. Forward and reverse individual gene sequences were aligned using ClustalW and sequencing errors were manually corrected. A consensus sequence was obtained and trimmed to an appropriate length. Gene sequences of each strain were then combined to produce concatenated sequences for MLSA. Phylogenetic analyses were performed in MEGA6 (Tamura et al., 2013) using the Neighbor Joining method and the maximum composite likelihood model with a bootstrap of 1000 replications. Sequences of the seven genes, from *V. aestuarianus* 02/041, *V. ordalii* 12B09 and *V. penaeicida* AQ115, were kindly provided from Genomic of *Vibrio*

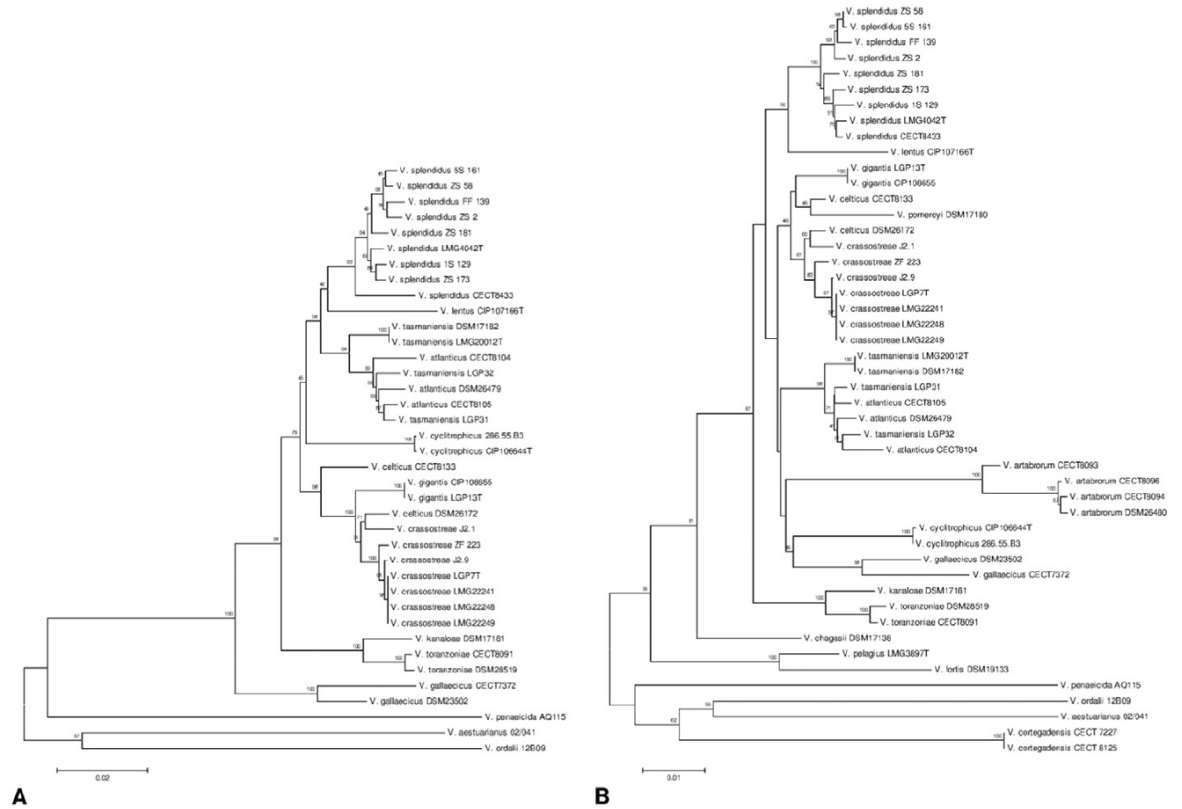


Fig. 1. Phylogenetic trees of reference strains based on the Neighbor Joining method using the concatenated sequences of 7 genes (A) *atpA*, *ftsZ*, *gapA*, *gyrB*, *mreB*, *rpoD*, and *topA* or 5 genes Δ*gapA*/*gyrB* (B). At each node, the percentage value corresponding to 1000 bootstrap replications is given.

Research Department (CNRS Roscoff, France) and used as Splendidus clade outsiders.

2.4. Experimental infections with field strains

A healthy adult mussel batch of *Mytilus edulis* from Normandy coast was used in experimental infections. Mussels were acclimatized to aquarium conditions for a minimum of 72 h in seawater beakers at around 19 °C under oxygenation conditions. After an hour of emersion in order to boost successively the filtering capacities, animals were then anaesthetized by bathing 2 h in a solution containing 50 g/L of magnesium chloride dissolved in two-thirds of distilled water and one third of seawater. The 23 strains isolated from mussels during and outside mortality events were tested with experimental infections. For each field strain, 100 μL of bacteria suspended in sterile artificial seawater were injected into the adductor muscle of a set of 5 animals at a concentration of 10^4 UFC/μL. A negative control was carried out with 5 animals injected with sterile artificial seawater (23 g/L NaCl; 1.5 g/L KCl; 1.23 g/L $MgSO_4$,$7H_2O$; $CaCl_2$ 0.3 g/L).

Mortality was monitored every day for 7 days. Dying animals were taken daily to isolate *V. splendidus*-related strains from mussel tissue homogenates as described in Section 2.2. An individual gene tree was constructed on sequences of *V. splendidus*-related strains isolated from all batches suffering mortality and on sequences of inoculated strains. When 100% homology in gene sequences was found between isolated and inoculated strains, a MLSA tree was done on isolates from batches suffering mortality higher than 30% and from healthy mussels sampled in the control test.

3. Results

3.1. MLSA validation

Phylogenetic tree based on the sequencing of 7-gene concatemers clearly separate the different species which composed the *V. splendidus* clade with high bootstrap values (>80%) except for the two *V. celticus* strains situated in separate branches (Fig. 1A). However, *V. artabrorum*, *V. chagasii*, *V. fortis*, *V. hemicentroti*, *V. pelagius*, *V. pomeroyi* and *V. cortegadensis* could not be classified using the 7-gene tree because amplification failed for 6 genes of *V. hemicentroti* and for one of the other species: *gapA* was not amplified for *V. artabrorum* and *gyrB* for the others. Individual gene trees were constructed to classify all strains and to identify the best gene combination for MLSA. Only the *mreB* tree clearly separated the different clusters as well as the 7-gene tree even if lower bootstrap values were obtained. Regarding the other single trees, some species were misclassified in wrong clusters. For instance, in the *gapA* tree, the strain *V. crassostreae* J2.1 was allied with the *tasmaniensis* cluster instead of the cluster composed of *V. crassostreae* strains. Moreover, some single trees as *atpA* and *rpoD* were not able to discriminate between *gigantis* and *crassostreae* clusters. A Δ*gapA*/*gyrB* 5-gene tree was constructed to get a reliable tool able to both obtain well-separated clusters and include all species (Fig. 1B).

In the 7-gene and 5-gene trees, *V. atlanticus* was very closely related to *V. tasmaniensis* and both *V. tasmaniensis* strains LGP31 and LGP32 were incorporated in the *atlanticus* group. In both trees, the *V. gallaecicus* strains formed within the clade a well separated branch, corroborated by a bootstrap value >98%. *V. pomeroyi*, *V. pelagius*, *V. chagasii* and *V. fortis* could be classified only with the 5-gene tree. The two latter

Table 2
Vibrio splendidus-related strains isolated from blue mussels: origin, health status, MLSA classification and mortality rate obtained after 7 days from strain inoculation.

Strain	Origin	Health status at sampling[a]	Number of successful sequenced genes for MLSA identification[b]	MLSA classification[c]	Mortality rate obtained after 7 days from strain inoculation (%)
M1A	Chausey, Manche	H	5	Near cluster *artabrorum*	20
M1B	Chausey, Manche	H	5	Near cluster *artabrorum*	0
M2B	Chausey, Manche	D	5	Cluster *splendidus*	0
M2C	Chausey, Manche	D	5	Cluster *splendidus*	20
M3B	Charente-Maritime	D	5	Cluster *tasmaniensis*	0
M3E	Charente-Maritime	D	5	Cluster *tasmaniensis*	0
M3F	Charente-Maritime	D	5	Cluster *splendidus*	20
M3H	Charente-Maritime	D	5	Cluster *splendidus*	80
M4A	Annoville, Manche	H	5	Cluster *tasmaniensis*	20
M8E	Saint Aubin, Calvados	H	5	Cluster *artabrorum*	0
M8F	Saint Aubin, Calvados	H	5	Cluster *artabrorum*	0
M8H	Saint Aubin, Calvados	H	5	Cluster *tasmaniensis*	0
M9H	Sainte Adresse, Seine-Maritime	H	5	Cluster *tasmaniensis*	0
M10B	Sainte Adresse, Seine-Maritime	H	5	Cluster *splendidus*	0
M10E	Sainte Adresse, Seine-Maritime	H	5	Cluster *splendidus*	0
M10F	Sainte Adresse, Seine-Maritime	H	5	Cluster *splendidus*	0
M11A	Ouistreham, Calvados	H	1 (*ftsZ*, *mreB*, *rpoD*, *topA*)	Outside the *V. splendidus* group (*V. rumoiensis*)	0
M11C	Ouistreham, Calvados	H	1 (*ftsZ*, *mreB*, *rpoD*, *topA*)	Outside the *V. splendidus* group (*V. rumoiensis*)	0
M12B	Saint Vaast la Hougue, Manche	H	5	Cluster *splendidus*	0
M12D	Saint Vaast la Hougue, Manche	H	5	Cluster *artabrorum*	0
M12J	Saint Vaast la Hougue, Manche	H	5	Cluster *artabrorum*	0
M13C	Ouistreham, Calvados	H	5	Near cluster *gallaecicus*	0
M14D	Saint Aubin sur mer, Calvados	H	5	Cluster *artabrorum*	0

[a] H: healthy, D: dying.
[b] In brackets: no PCR product for this (these) gene(s).
[c] In brackets: identification with Nucleotide Blast search (NCBI).

Table 3
Experimental infections with the M3H field strain.

Experiment	Mortality rate (%)	Number of relevant tested *Vibrio splendidus* related strains[a]	Number of isolated strains with 100% homology with M3H[b]
Initial (Test 0)	80	14	3
Repeat 1	0	–	–
Repeat 2	20	7	0
Repeat 3	60	10	3
Repeat 4	20	4	1
Control	0	9	0

[a] Isolated from dying animals except for the control trial.
[b] According to the 5-gene MLSA.

species were found closely related to each other. The *V. celticus* strains were not clustered together with both trees. In the 5-gene tree, the CECT8133 strain was classified closed to *V. pomeroyi* whereas the DSM26172 strain was allied with *crassostreae* cluster. *V. hemicentroti* could be classified only with the *atpA* tree and was placed far from the Splendidus clade. Finally, *V. cortegadensis*, resulted phylogenetically more distant through all the multi and single-gene analyses. The PCR2, specific to Splendidus clade, was carried out for *V. hemicentroti* and *V. cortegadensis* and very late Ct values were obtained for both strains.

Compared to the 7-gene tree, the Δ*gapA*/*gyrB* 5-gene tree allowed to include all the species with a good strain identification and was used subsequently for the characterization of mussel isolates.

3.2. Characterization of mussel isolates

A total of 23 *V. splendidus*-related strains were isolated from *M. edulis* taken on French farms during the summer 2014: 17 from healthy mussels and 6 from dying animals (Table 2). Ct values obtained for all strains with Real-Time PCRs were 14.9 ± 3 for *Vibrio* spp. (PCR1). Concerning the PCR2, specific to *V. splendidus*-related strains, early Ct values were obtained for all isolates except for three strains (M11A, M11C and M13C). 5-gene amplification succeeded for all strains, except for M11A and M11C. 38% of isolates analysed with the 5-gene MLSA were classified in the *V. splendidus* cluster, 24% in the *V. tasmaniensis* cluster and 24% in the *V. artabrorum* one, whereas 14% were placed on distinct branches (Table 2). Within these, M1A and M1B formed a separate branch with affinities to the *V. artabrorum* cluster, while M13C was closer to *V. gallaecicus*.

Because of the unsuccessful 5-gene amplification, only the *atpA* tree could be constructed for the two unclassified strains (M11A and M11C) and it sorted them outside the Splendidus clade. A Blast search using NCBI database was done on *atpA* sequences of both strains and identified them as *Vibrio rumoiensis* with a 99% identity rate.

3.3. Strain pathogenicity trials

The 23 field strains from healthy or dying animals were all tested by injection into the adductor muscle of a healthy adult mussel batch. Mortality was observed for 5 out of the 23 tested strains, with rates of 20% except for the M3H strain which have induced 80% of dead animals (Table 2). Among the 5 strains, two were originally isolated from healthy batches (M1A, M4A).

A *mreB* tree was constructed for 36 *V. splendidus*-related strains isolated from dying animals, previously infected with the above 5 strains, and collected during the 7-day experimentation. On 22 relevant strains from the 4 batches in which mortality rates were 20%, no isolate was found to be identical to the original inoculated strain, suggesting that they could be considered as innocuous. Conversely, 14 *V. splendidus*-related strains were isolated from moribund mussels injected with the M3H strain and 3 of them had 100% homology with the *mreB* gene of the parental strain. A 5-gene tree was carried out on these 3 strains and confirmed the result obtained with the *mreB* tree. To affirm the virulence of the M3H strain, the same experiment was repeated 4 times and mortality rates varied between 0 and 60% (Table 3). Four strains isolated from dying animals were found 100% identical to M3H according to the 5-gene tree: 3 strains from the test 3 and 1 strain from the test 4 with mortality rates at 60 and 20% respectively (Fig. 2). No strain was found equivalent to M3H in the negative control and in the test 2 in which mortality rate was 20%.

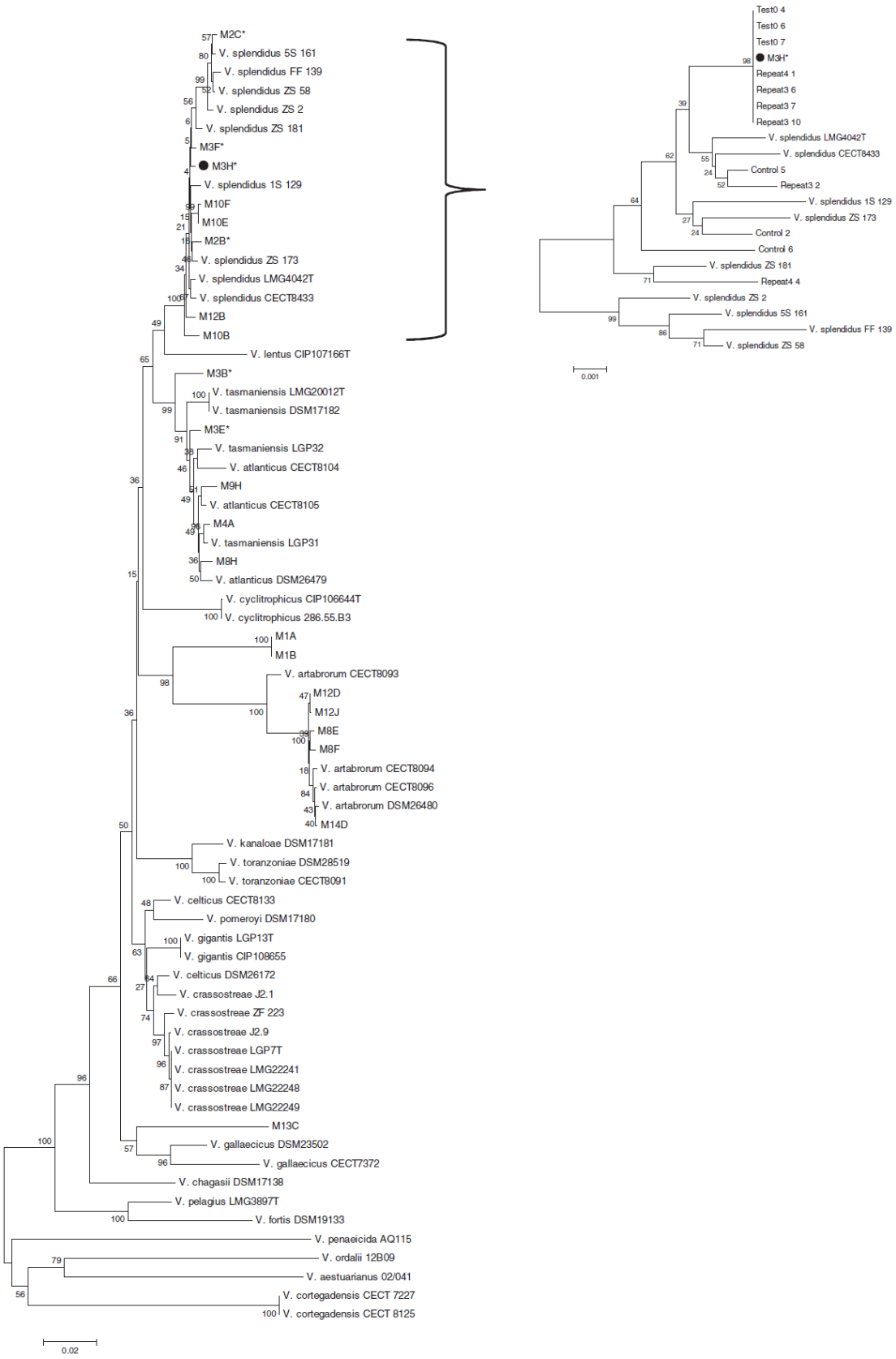
4. Discussion

V. splendidus-related strains represent the prevalent species composing the mussel *Mytilus* sp. normal microflora (Kwan and Bolch, 2015). Members of this group are also well-known to be pathogenic for bivalve molluscs, but during mortality events two problems remained to be resolved to give an accurate diagnosis response. Firstly, vibrios from the Splendidus clade are closely related and a potent tool was needed to obtain a correct identification of them. Secondly, a comparison between individual microflora during and outside mortality events was suggested to understand the microbiological dynamics during mortalities. Our present work revealed that the 5-gene MLSA tree is an accurate tool to discriminate species within the Splendidus clade. Previous studies have already worked on MLSA schemes including all the family *Vibrionaceae* (Gabriel et al., 2014; Sawabe et al., 2007, 2013; Thompson et al., 2005). Only a member of each *V. splendidus*-related strain has been tested in these studies. Another recent work showed that the *fur* gene could be a powerful discriminative phylogenetic marker to identify *Vibrionaceae* species (Machado and Gram, 2015). Nevertheless, within the Splendidus clade, strains were not clearly separated using the single *fur* gene sequence analysis. Phylogenetic analyses were previously performed on *V. splendidus*-related strains based on *gyrB* sequences (Le Roux et al., 2004). This gene appeared to be a good marker within the group, however it poorly discriminated several species *i.e. V. kanaloae* LMG20539^T and *V. tasmaniensis* LMG20012^T.

During the present study, 7 housekeeping genes were tested (*atpA*, *ftsZ*, *gapA*, *gyrB*, *mreB*, *rpoD* and *topA*) to develop an MLSA tool allowing an accurate identification of strains from the Splendidus clade. In fact, the use of a single gene analysis resulted unreliable for the identification of strains from this clade. The classification provided by *atpA*, *gapA* and *gyrB* analyses was unclear because of the incapacity of these genes to discriminate between several species of the group. Besides, recombination might be responsible for conflicting phylogenetic signals whatever the single-gene tree considered. Within all studied targets, *mreB* was the most discriminant gene and thus was superior to the others for the identification of closely related strains among the clade.

Clusters of species were in general agreement with the 7-gene and the Δ*gapA*/*gyrB* 5-gene trees. Despite bootstrap values a little lower with 5-gene tree, this analysis allowed to include the totality of *V. splendidus*-related strains in the tree, except *V. hemicentroti*. About this strain, it is interesting to highlight the absence of amplification of six genes, suggesting diversity in the sequences of these target regions and its exclusion from the Splendidus clade with *atpA* analysis. Unlike *V. hemicentroti*, 6 out of the 7 studied genes could be analysed for *V. cortegadensis* but these species were also classified outside the Splendidus clade. Results of PCR2 confirmed the exclusion of *V.*

Fig. 2. Phylogenetic tree of reference and isolated strains based on the Neighbor Joining method using the concatenated sequences of 5 genes *atpA*, *ftsZ*, *mreB*, *rpoD* and *topA*. Strains indicated with an asterisk were isolated from dying animals. On the right of the figure, the recovered strains isolated after experimental injections of M3H were added to the tree. At each node, the percentage value corresponding to 1000 bootstrap replications is given.


M2C*
V. splendidus 5S 161
V. splendidus FF 139
V. splendidus ZS 58
V. splendidus ZS 2
V. splendidus ZS 181
M3F*
M3H*
V. splendidus 1S 129
M10F
M10E
M2B*
V. splendidus ZS 173
V. splendidus LMG4042T
V. splendidus CECT8433
M12B
M10B
V. lentus CIP107166T
M3B*
V. tasmaniensis LMG20012T
V. tasmaniensis DSM17182
M3E*
V. tasmaniensis LGP32
V. atlanticus CECT8104
M9H
V. atlanticus CECT8105
M4A
V. tasmaniensis LGP31
M8H
V. atlanticus DSM26479
V. cyclitrophicus CIP106644T
V. cyclitrophicus 286.55.B3
M1A
M1B
V. artabrorum CECT8093
M12D
M12J
M8E
M8F
V. artabrorum CECT8094
V. artabrorum CECT8096
V. artabrorum DSM26480
M14D
V. kanaloae DSM17181
V. toranzoniae DSM28519
V. toranzoniae CECT8091
V. celticus CECT8133
V. pomeroyi DSM17180
V. gigantis LGP13T
V. gigantis CIP108655
V. celticus DSM26172
V. crassostreae J2.1
V. crassostreae ZF 223
V. crassostreae J2.9
V. crassostreae LGP7T
V. crassostreae LMG22241
V. crassostreae LMG22248
V. crassostreae LMG22249
M13C
V. gallaecicus DSM23502
V. gallaecicus CECT7372
V. chagasii DSM17138
V. pelagius LMG3897T
V. fortis DSM19133
V. penaeicida AQ115
V. ordalii 12B09
V. aestuarianus 02/041
V. cortegadensis CECT 7227
V. cortegadensis CECT 8125
0.02
Test0 4
Test0 6
Test0 7
M3H*
Repeat4 1
Repeat3 6
Repeat3 7
Repeat3 10
V. splendidus LMG4042T
V. splendidus CECT8433
Control 5
Repeat3 2
V. splendidus 1S 129
V. splendidus ZS 173
Control 2
Control 6
V. splendidus ZS 181
Repeat4 4
V. splendidus ZS 2
V. splendidus 5S 161
V. splendidus FF 139
V. splendidus ZS 58
0.001

hemicentroti and *V. cortegadensis* outside the Splendidus clade. With the exception of *V. pomeroyi*, the strains with an unsuccessful amplification of one gene (*V. artabrorum*, *V. chagasii*, *V. fortis* and *V. pelagius*) were divided from the other clusters by a high bootstrap value ˜90%, evidenced in Fig. 1B. A recent study called into question the classification of *V. fortis* within the group (Nasfi et al., 2015) and regarding our results, also *V. pelagius* and *V. chagasii* seemed to be far from the other *V. splendidus*-related species.

The exact location of *V. celticus* CECT8133 strain in 5-gene trees resulted however skewed because of *topA* gene sequence suggesting a putative recombination event.

Interestingly, *V. tasmaniensis* LGP31 and LGP32 resulted identified as *V. atlanticus* whatever the gene considered, and *V. crassostreae* J2.1 clustered mostly with *V. celticus* DSM26172 suggesting the need of further investigations for their taxonomic assignment.

The 5-housekeeping gene MLSA approach developed in this study appeared to be a useful and speed technique to discriminate *V. splendidus*-related strains isolated both during and outside mortality outbreaks. Only two strains failed to be classified with the 5-gene tree because of the lack of 4 amplified genes. A Blast method identified them as *V. rumoiensis* which is far from the Splendidus clade, thus questioning the specificity of the real-time PCRs targeting the 16S rRNA gene. The same results were obtained in a recent work where 100 out of 435 isolates have discordant results between 16S rRNA PCR targeting the Splendidus clade and VNTR or Variable Number of Tandem Repeats analysis (Nasfi et al., 2015).

Our results revealed a large diversity of isolates belonging to Splendidus clade whatever the health status of analysed mussels. Recently, a high diversity of species from this group was also underlined in an Australian mussel hatchery without mortality outbreaks (Kwan and Bolch, 2015). In our study, the number of analysed dying batches was very lower than the healthy ones which made any statistic comparison difficult between the two groups. The large diversity observed might be due to the variability of sampling location. For instance, two batches (M8, M14) were together sampled in Saint-Aubin (Calvados) at different times and both showed the presence of strains allied with the *V. artabrorum* cluster.

However, the predominance of strains belonging to the Splendidus clade was demonstrated in samples taken from different mortality events. We might hypothesize that either these strains could be pathogenic agents, responsible for mussel mortalities, or either they could invade the necrotic mussel tissues because of their natural abundance in mussel environment. Experimental infections are needed to affirm or exclude, with certainty, the virulence of these strains. In our study, the *V. splendidus* M3H strain, originated from a dying batch sampled in Charente-Maritime, seemed to be pathogenic to mussels. Indeed, this strain was able to induce mortality over than 60% when injected to healthy animals and was then recovered in dying batches showing high mortality rates. So far, little information has been published on mussel pathogenic *Vibrio*. The moderate pathogenicity of *V. splendidus* on adult mussels has already been proved but high concentrations of bacteria, combined with bad environmental conditions, were necessary to induce mortality in experimental infections, suggesting a high resistance of mussels to bacterial pathogens (Romero et al., 2014). Conversely, the *V. tasmaniensis* LGP32 strain previously recognized as responsible for *C. gigas* mortalities was also tested on experimental infections with mussels and was demonstrated as nonpathogenic to *M. galloprovincialis* but induced stressful conditions on tissues (Balbi et al., 2013). Recently, a pathogenic *Vibrio* affiliated to *V. splendidus*/*V. hemicentroti* type strains has been evidenced to be responsible for alteration of *Mytilus edulis* hemocyte functions (Ben Cheikh et al., 2016). Its virulence was proved by several experimental strain injections but the recovery of isolates, identical to the injected strain, was not realized in dying animals. In accordance with Koch's postulates, we can affirm in our study the pathogenicity of the M3H strain, allied with the *V. splendidus* cluster.

In conclusion, our results showed that the 5-gene MLSA developed in this work is a useful tool to classify species among the Splendidus clade. This study revealed a large diversity of strains among this clade in mussel animals, whatever their health status. However, experimental trials have revealed the presence of a pathogenic strain isolated from a moribund mussel batch, *V. splendidus* M3H, which has been proved to induce mortality *in vivo*. Further investigations should be now conducted to check the M3H pathogenicity and pathogenesis in various healthy mussel batches and under various conditions.

Acknowledgments

We thank IFREMER station in la Tremblade and Dr Frédérique LEROUX (Genomic of Vibrio-Station Biologique de Roscoff) for giving us *Vibrio* strains. This study would not have been possible without the contribution of French mussel farmers and the CRC Normandie-Mer du Nord.

References

Balbi, T., Fabbri, R., Cortese, K., Smerilli, A., Ciacci, C., Grande, C., Vezzulli, L., Pruzzo, C., Canesi, L., 2013. Interactions between *Mytilus galloprovincialis* hemocytes and the bivalve pathogens *Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32. Fish Shellfish Immunol. 35, 1906–1915. http://dx.doi.org/10.1016/j.fsi.2013.09.027.

Beaz-Hidalgo, R., Doce, A., Pascual, J., Toranzo, A.E., Romalde, J.L., 2009. *Vibrio gallaecicus* sp. nov. isolated from cultured clams in north-western Spain. Syst. Appl. Microbiol. 32, 111–117. http://dx.doi.org/10.1016/j.syapm.2008.12.002.

Beaz-Hidalgo, R., Balboa, S., Romalde, J.L., Figueras, M.J., 2010a. Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs: *Vibrio* spp., bivalve molluscs, pathogens. Environ. Microbiol. Rep. 2, 34–43. http://dx.doi.org/10.1111/j.1758-2229.2010.00135.x.

Beaz-Hidalgo, R., Diéguez, A.L., Cleenwerck, I., Balboa, S., Doce, A., de Vos, P., Romalde, J.L., 2010b. *Vibrio celticus* sp. nov., a new *Vibrio* species belonging to the splendidus clade with pathogenic potential for clams. Syst. Appl. Microbiol. 33, 311–315. http://dx.doi.org/10.1016/j.syapm.2010.06.007.

Bechemin, C., Soletchnik, P., Polsenaere, P., Le Moine, O., Pernet, F., Protat, M., Fuhrmann, M., Quere, C., Goulitquer, S., Corporeau, C., Renault, T., Lapegue, S., Travers, M.-A., Morga, B., Garriques, M., Garcia, C., Haffner, P., Dubreuil, C., Faury, N., Baillon, L., Baud, J.-P., 2014. Surmortalités de la moule bleue *Mytilus edulis* dans les Pertuis Charentais (mars 2014). http://archimer.ifremer.fr/doc/00229/34022/.

Ben Cheikh, Y., Travers, M.-A., Morga, B., Godfrin, Y., Rioult, D., Le Foll, F., 2016. First evidence for a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities. Dev. Comp. Immunol. 57, 107–119. http://dx.doi.org/10.1016/j.dci.2015.12.014.

De Decker, S., Saulnier, D., 2011. Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: evidence of early infection and down-expression of immune-related genes. Fish Shellfish Immunol. 30, 691–699. http://dx.doi.org/10.1016/j.fsi.2010.12.017.

Dieguez, A.L., Beaz-Hidalgo, R., Cleenwerck, I., Balboa, S., de Vos, P., Romalde, J.L., 2011. *Vibrio atlanticus* sp. nov. and *Vibrio artabrorum* sp. nov., isolated from the clams *Ruditapes philippinarum* and *Ruditapes decussatus*. Int. J. Syst. Evol. Microbiol. 61, 2406–2411. http://dx.doi.org/10.1099/ijs.0.025320-0.

Faury, N., 2004. *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). Int. J. Syst. Evol. Microbiol. 54, 2137–2140. http://dx.doi.org/10.1099/ijs.0.63232-0.

Gabriel, M.W., Matsui, G.Y., Friedman, R., Lovell, C.R., 2014. Optimization of multilocus sequence analysis for identification of species in the genus Vibrio. Appl. Environ. Microbiol. 80, 5359–5365. http://dx.doi.org/10.1128/AEM.01206-14.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., Nicolas, J.-L., 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microb. Ecol. 53, 187–196. http://dx.doi.org/10.1007/s00248-006-9061-9.

Gay, M., Berthe, F.C., Le Roux, F., 2004a. Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. Dis. Aquat. Org. 59, 49. http://dx.doi.org/10.3354/dao059049.

Gay, M., Renault, T., Pons, A.-M., Le Roux, F., 2004b. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. Dis. Aquat. Org. 62, 65–74. http://dx.doi.org/10.3354/dao062065.

Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F.L., Swings, J., 2005. Opinion: re-evaluating prokaryotic species. Nat. Rev. Microbiol. 3 (9), 733–739. http://dx.doi.org/10.1038/nrmicro1236.

Gomez-Leon, J., Villamil, L., Lemos, M.L., Novoa, B., Figueras, A., 2005. Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. Appl. Environ. Microbiol. 71, 98–104. http://dx.doi.org/10.1128/AEM.71.1.98-104.2005.

Hedlund, B.P., Staley, J.T., 2001. *Vibrio cyclotrophicus* sp. nov., a polycyclic aromatic hydrocarbon (PAH)-degrading marine bacterium. Int. J. Syst. Evol. Microbiol. 51, 61–66. http://dx.doi.org/10.1099/00207713-51-1-61.

Kim, D., Baik, K.S., Hwang, Y.S., Choi, J.-S., Kwon, J., Seong, C.N., 2013. *Vibrio hemicentroti* sp. nov., an alginate lyase-producing bacterium, isolated from the gut microflora of sea urchin (*Hemicentrotus pulcherrimus*). Int. J. Syst. Evol. Microbiol. 63, 3697–3703. http://dx.doi.org/10.1099/ijs.0.047951-0.

Kwan, T., Bolch, C., 2015. Genetic diversity of culturable *Vibrio* in an Australian blue mussel *Mytilus galloprovincialis* hatchery. Dis. Aquat. Org. 116, 37–46. http://dx.doi.org/10.3354/dao02905.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M., Poulet, S.A., 2001. A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the bay of Morlaix (North Brittany, France). Dis. Aquat. Org. 46, 139–145. http://dx.doi.org/10.3354/dao046139.

Lasa, A., Diéguez, A.L., Romalde, J.L., 2013. *Vibrio toranzoniae* sp. nov., a new member of the splendidus clade in the genus *Vibrio*. Syst. Appl. Microbiol. 36, 96–100. http://dx.doi.org/10.1016/j.syapm.2012.11.005.

Le Roux, F., Gay, M., Lambert, C., Waechter, M., Poubalanne, S., Chollet, B., Nicolas, J.-L., Berthe, F., 2002. Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. Aquat. Living Resour. 15, 251–258.

Le Roux, F., Gay, M., Lambert, C., Nicolas, J.-L., Gouy, M., Berthe, F.C.J., 2004. Phylogenetic study and identification of *Vibrio splendidus*-related strains based on gyrB gene sequences. Dis. Aquat. Org. 58, 143. http://dx.doi.org/10.3354/dao058143.

Liu, R., Qiu, L., Yu, Z., Zi, J., Yue, F., Wang, L., Zhang, H., Teng, W., Liu, X., Song, L., 2013. Identification and characterisation of pathogenic *Vibrio splendidus* from yesso scallop (*Patinopecten yessoensis*) cultured in a low temperature environment. J. Invertebr. Pathol. 114, 144–150. http://dx.doi.org/10.1016/j.jip.2013.07.005.

Machado, H., Gram, L., 2015. The *fur* gene as a new phylogenetic marker for Vibrionaceae species identification. Appl. Environ. Microbiol. 81, 2745–2752. http://dx.doi.org/10.1128/AEM.00058-15.

Macián, M.C., Ludwig, W., Aznar, R., Grimont, P.A., Schleifer, K.H., Garay, E., Pujalte, M.J., 2001. *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. Int. J. Syst. Evol. Microbiol. 51, 1449–1456. http://dx.doi.org/10.1099/00207713-51-4-1449.

Myrand, B., Guderley, H., Himmelman, J.H., 2000. Reproduction and summer mortality of blue mussels *Mytilus edulis* in the Magdalen Islands, southern gulf of St. Lawrence. Mar. Ecol. Prog. Ser. 197, 193–207. http://dx.doi.org/10.3354/meps197193.

Nasfi, H., Travers, M.A., de Lorgeril, J., Habib, C., Sannie, T., Sorieul, L., Gerard, J., Avarre, J.C., Haffner, P., Tourbiez, D., Renault, T., Furones, D., Roque, A., Pruzzo, C., Cheslett, D., Gdoura, R., Vallaeys, T., 2015. A European epidemiological survey of *Vibrio splendidus* clade shows unexplored diversity and massive exchange of virulence factors. World J. Microbiol. Biotechnol. http://dx.doi.org/10.1007/s11274-015-1800-y.

Peperzak, L., Poelman, M., 2008. Mass mussel mortality in The Netherlands after a bloom of *Phaeocystis globosa* (prymnesiophyceae). J. Sea Res. 60, 220–222. http://dx.doi.org/10.1016/j.seares.2008.06.001.

Prieur, D., Mevel, G., Nicolas, J.-L., Plusquellec, A., Vigneulle, M., 1990. Interactions Between Bivalve Molluscs and Bacteria in the Marine Environment. Oceanography and Marine Biology - An Annual Review pp. 277–352.

Pujalte, M.J., Ortigosa, M., Macián, M.C., Garay, E., 2010. Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. Int. Microbiol. 2, 259–266.

Romalde, J.L., Dieguez, A.L., Lasa, A., Balboa, S., 2014. New *Vibrio* species associated to molluscan microbiota: a review. Front. Microbiol. 4. http://dx.doi.org/10.3389/fmicb.2013.00413.

Romero, A., Costa, M., Forn-Cuni, G., Balseiro, P., Chamorro, R., Dios, S., Figueras, A., Novoa, B., 2014. Occurrence, seasonality and infectivity of *Vibrio* strains in natural populations of mussels *Mytilus galloprovincialis*. Dis. Aquat. Org. 108, 149–163. http://dx.doi.org/10.3354/dao02701.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., Garcia, C., 2010. A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. Microb. Ecol. 59, 787–798. http://dx.doi.org/10.1007/s00248-009-9620-y.

Sawabe, T., Kita-Tsukamoto, K., Thompson, F.L., 2007. Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. J. Bacteriol. 189, 7932–7936. http://dx.doi.org/10.1128/JB.00693-07.

Sawabe, T., Ogura, Y., Matsumura, Y., Feng, G., Amin, A.R., Mino, S., Nakagawa, S., Sawabe, T., Kumar, R., Fukui, Y., Satomi, M., Matsushima, R., Thompson, F.L., Gomez-Gil, B., Christen, R., Maruyama, F., Kurokawa, K., Hayashi, T., 2013. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. Front. Microbiol. 4. http://dx.doi.org/10.3389/fmicb.2013.00414.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A., Kämpfer, P., Maiden, M.C., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C., Whitman, W.B., 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52 (Pt. 3), 1043–1047. http://dx.doi.org/10.1099/00207713-52-3-1043.

Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., Muroga, K., 1998. *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. Dis. Aquat. Org. 33, 111–118. http://dx.doi.org/10.3354/dao033111.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. http://dx.doi.org/10.1093/molbev/mst197.

Thompson, F.L., 2003a. *Vibrio kanaloae* sp. nov., *Vibrio pomeroyi* sp. nov. and *Vibrio chagasii* sp. nov., from sea water and marine animals. Int. J. Syst. Evol. Microbiol. 53, 753–759. http://dx.doi.org/10.1099/ijs.0.02490-0.

Thompson, F.L., 2003b. *Vibrio fortis* sp. nov. and *Vibrio hepatarius* sp. nov., isolated from aquatic animals and the marine environment. Int. J. Syst. Evol. Microbiol. 53, 1495–1501. http://dx.doi.org/10.1099/ijs.0.02658-0.

Thompson, F.L., Gevers, D., Thompson, C.C., Dawyndt, P., Naser, S., Hoste, B., Munn, C.B., Swings, J., 2005. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl. Environ. Microbiol. 71, 5107–5115. http://dx.doi.org/10.1128/AEM.71.9.5107-5115.2005.

Travers, M.A., Morga, B., Garrigues, M., Garcia, C., Haffner, P., Dubreuil, C., Faury, N., Baillon, L., Renault, T., Le Roux, F., Bruto, M., 2014. Agents infectieux et mortalités de moules en 2014. Journées de la Surveillance de la santé des mollusques marins, 15-17 décembre 2014, Nantes, France.

Tsuchiya, M., 1983. Mass mortality in a population of the mussel *Mytilus edulis* L. caused by high temperature on rocky shores. J. Exp. Mar. Biol. Ecol. 66, 101–111. http://dx.doi.org/10.1016/0022-0981(83)90032-1.

Urakawa, H., Kita-Tsukamato, K., Ohwada, K., 1999. 16S rDNA restriction fragment length polymorphism analysis of psychrotrophic vibrios from Japanese coastal water. Can. J. Microbiol. 45, 1001–1007. http://dx.doi.org/10.1139/cjm-45-12-1001.

Wendling, C.C., Batista, F.M., Wegner, K.M., 2014. Persistence, seasonal dynamics and pathogenic potential of *Vibrio* communities from Pacific oyster hemolymph. PLoS ONE 9, e94256. http://dx.doi.org/10.1371/journal.pone.0094256.

Wertz, J.E., Goldstone, C., Gordon, D.M., Riley, M.A., 2003. A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. J. Evol. Biol. 16, 1236–1248. http://dx.doi.org/10.1046/j.1420-9101.2003.00612.x.

Yamamoto, S., Harayama, S., 1995. PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl. Environ. Microbiol. 61, 1104–1109.

Zeigler, D.R., 2003. Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int. J. Syst. Evol. Microbiol. 53, 1893–1900. http://dx.doi.org/10.1099/ijs.0.02713-0.

Monitoring of the environmental parameters

The collection of environmental data was conducted using various sources. For the off-shore, gulf, and intertidal environments we extracted the values of temperature and salinity from the databases delivered by the Copernicus Marine environment Monitoring Service (CMEMS). Downloaded files were then translated in an exploitable format using RStudio Desktop 1.0.136. However, this source was not suitable for the lagoon environments. Thus, we used the daily measurement data provided by the "Agenzia regionale per la prevenzione e protezione ambientale del Veneto" (ARPAV) and "Agenzia regionale per la protezione ambientale della Toscana" (ARPAT) for the environmental monitoring of the Caleri and Orbetello Lagoons respectively. Regrettably, for the sites of Varano, San Teodoro, and Olbia no public databases for daily monitoring were accessible from the regional ARPAs, with the exception of pluviometry, forcing to use only the measurements carried out during each of our samplings.

The environmental monitoring comprised also the evaluation of the net occlusion (lantern-nets and oyster bags). The presence of mud into the lantern compartments was also recorded.

Statistical analyses

A one-way ANOVA was generally applied to evaluate whether there were significant differences between the three different types of marine environment (open waters, lagoon and gulf) for growth. Since only two sites have been assigned to the "open waters" environment type, the significance of their differences was evaluated by t-test. A two-way ANOVA was performed to determine if there is an interaction effect between ploidy, site, and length. The Tukey test was used as *post-hoc* test to evaluate whether there were significant differences between the different sites. The effect of ploidy, site, allocation date, and year of the survey on mortality and prevalence of OsHV-1 was also evaluated through the use of Chi Square test. The Kruskal-Wallis test was applied to evaluate whether there were significant differences in viral and bacterial loads between the groups of samples, followed by a Tukey test as *post hoc*. Finally, difference in total bacterial load between apparently healthy and moribund individuals was evaluated with the Mann-Whitney test. Results with a p-value <0.05 were considered statistically significant. The statistical tests were performed using R software, version 3.3.1.

2.3.2. Results

At their arrival from the hatchery (in May, July, and September 2014; and in April and September 2015), the eight spat batches tested negative for OsHV-1.

During the survey, a total of 6214 and 6731 individuals were collected in 2014 and 2015 respectively.

Oyster growth

The initial length of oyster shell was about 7 mm. For the year 2014, at the end of the survey in middle October, the main growth was observed in the triploid individuals in Caleri and San Teodoro, with an average shell length of 83.53 and 83.17 mm respectively. The slowest growth was recorded in diploids in Caorle and Giulianova where the individual length reached the mean of 46.54 and 50.00 mm respectively. As expected, in all sites the final length of triploids was higher than diploids'.

The results of biometric measurements, site by site, are reported in Figure 48 (A to I). Interestingly, each site was characterised by a specific seasonal trend, such as Caorle where a constant growth was recorded or Varano where a peak of growth was observed in early autumn.

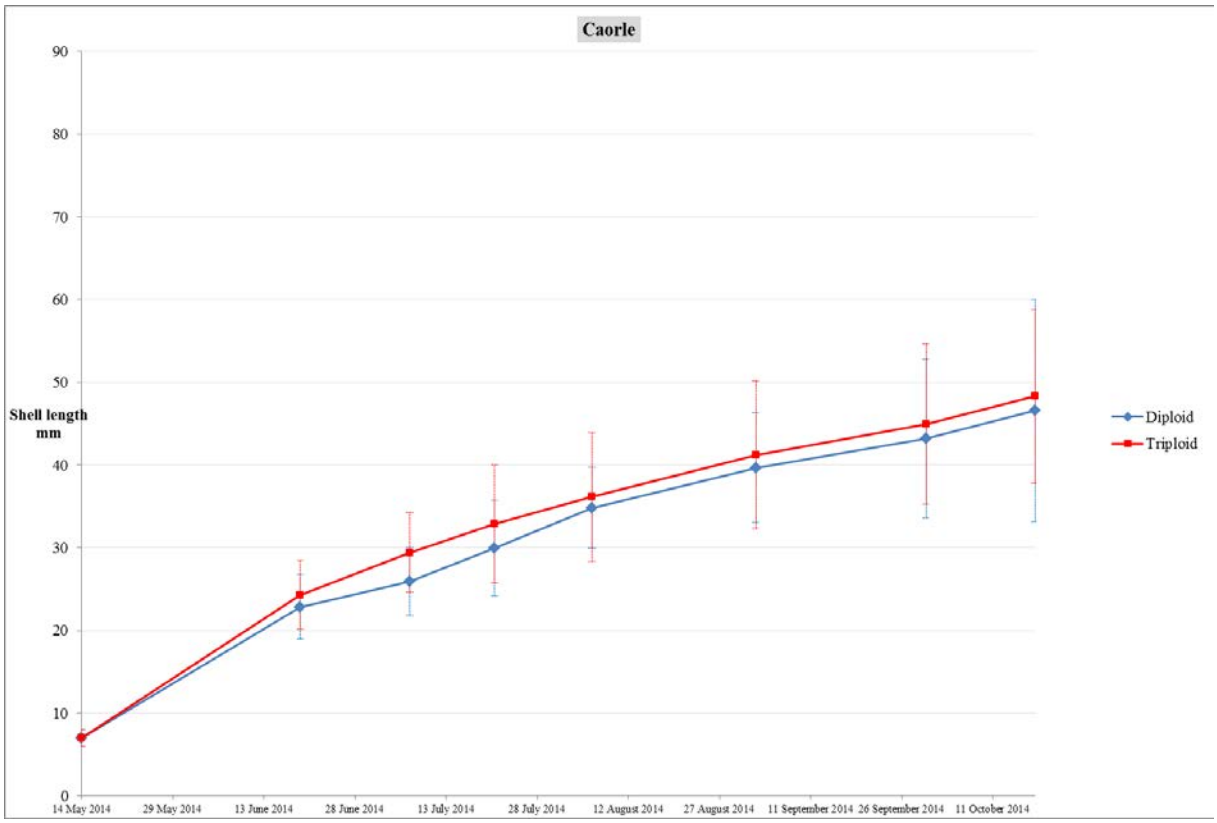


Fig. 48A Trend of the shell length of *C. gigas* individuals allocated in Caorle in May 2014, expressed in mm, in function of time and ploidy.

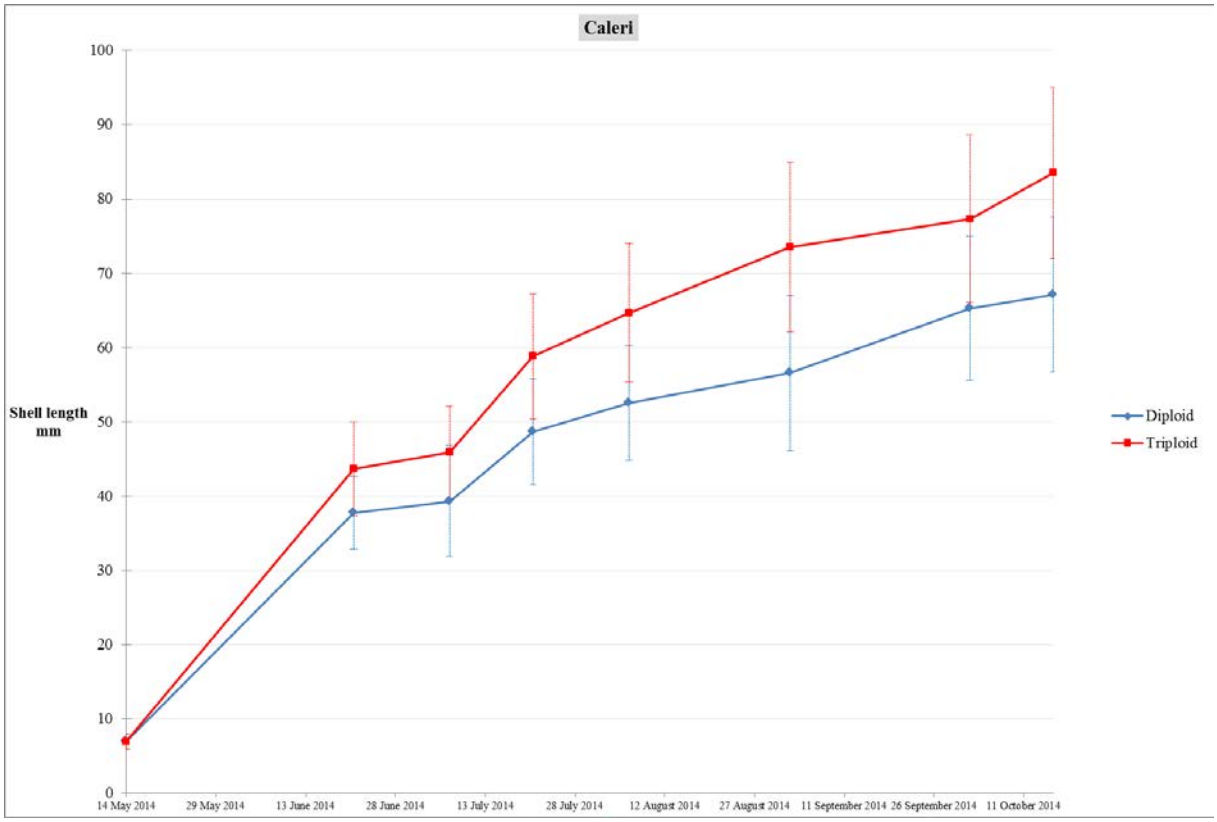


Fig. 48B Trend of the shell length of *C. gigas* individuals allocated in Caleri in May 2014, expressed in mm, in function of time and ploidy.

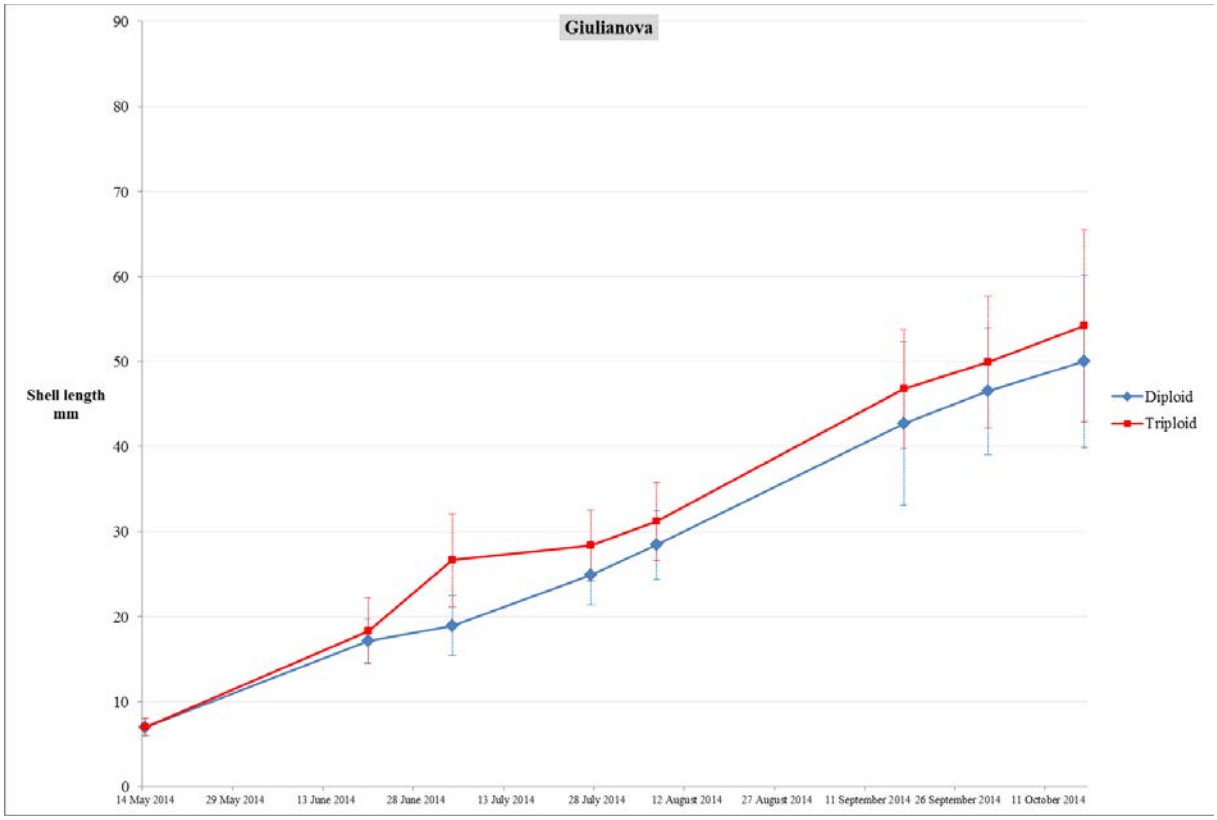


Fig. 48C Trend of the shell length of *C. gigas* individuals allocated in Giulianova in May 2014, in mm, in function of time and ploidy.

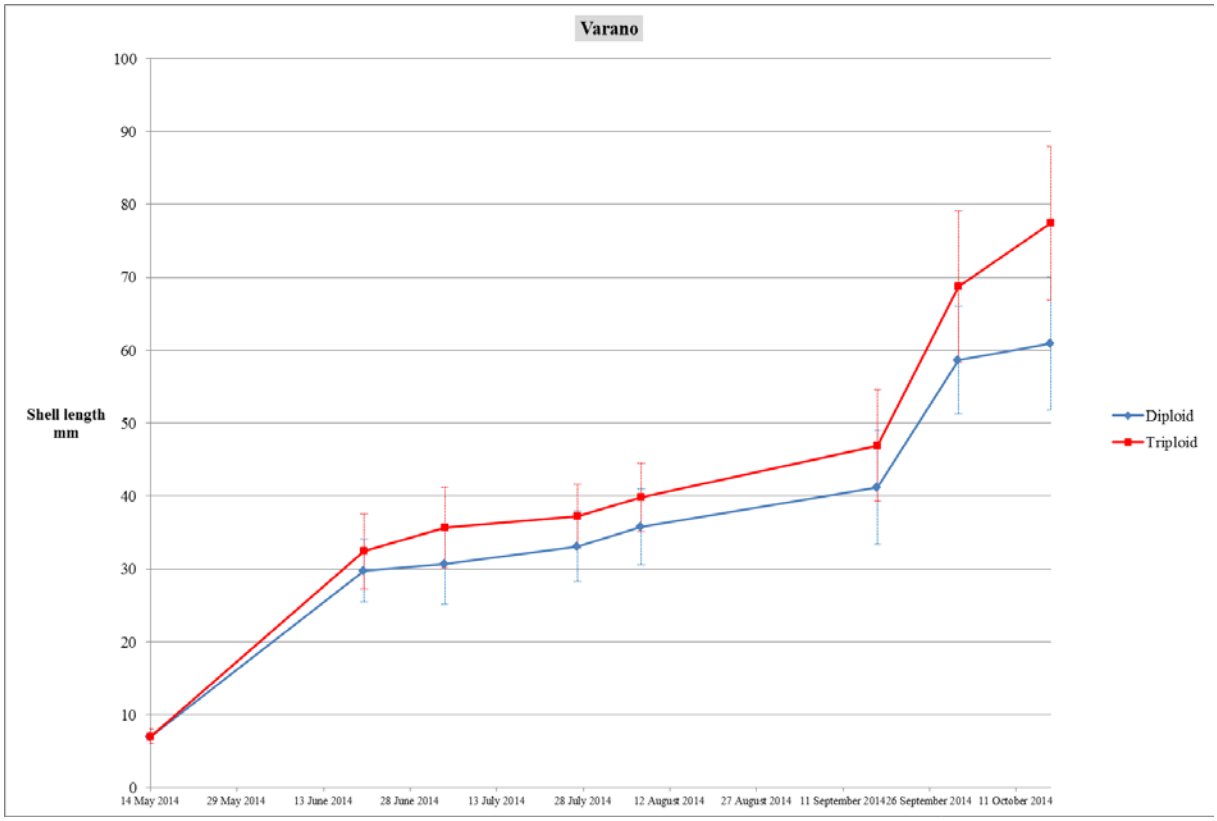


Fig. 48D Trend of the shell length of *C. gigas* individuals allocated in Varano in May 2014, in mm, in function of time and ploidy.

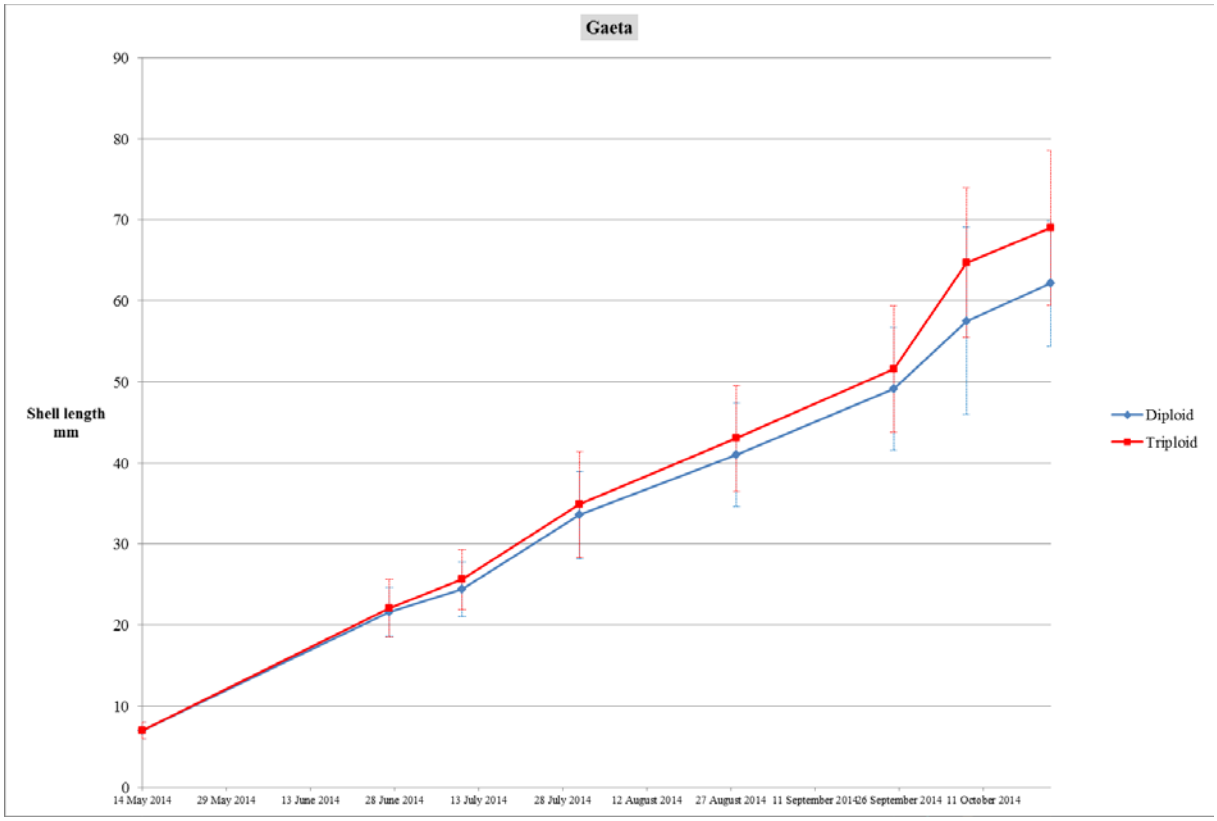


Fig. 48E Trend of the shell length of *C. gigas* individuals allocated in Gaeta in May 2014, expressed in mm, in function of time and ploidy.

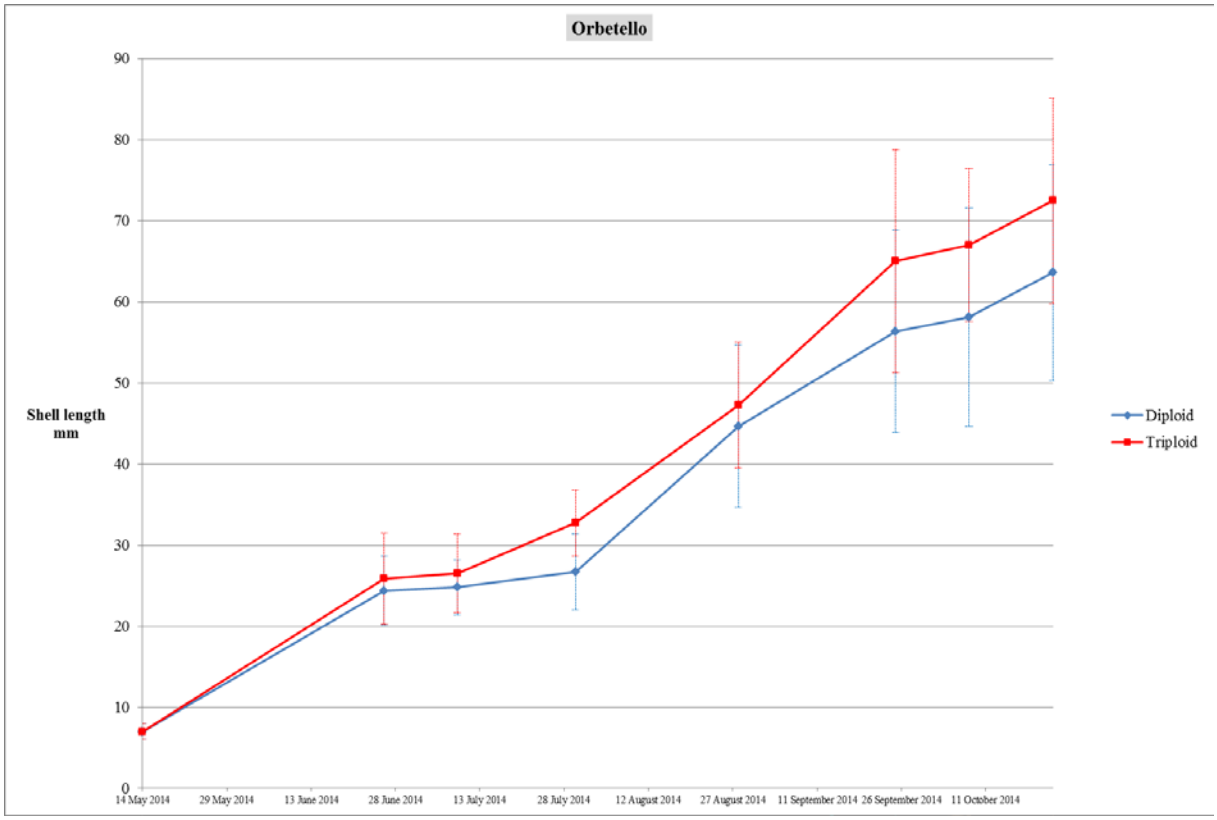


Fig. 48F Trend of the shell length of *C. gigas* individuals allocated in Orbetello in May 2014, in mm, in function of time and ploidy.

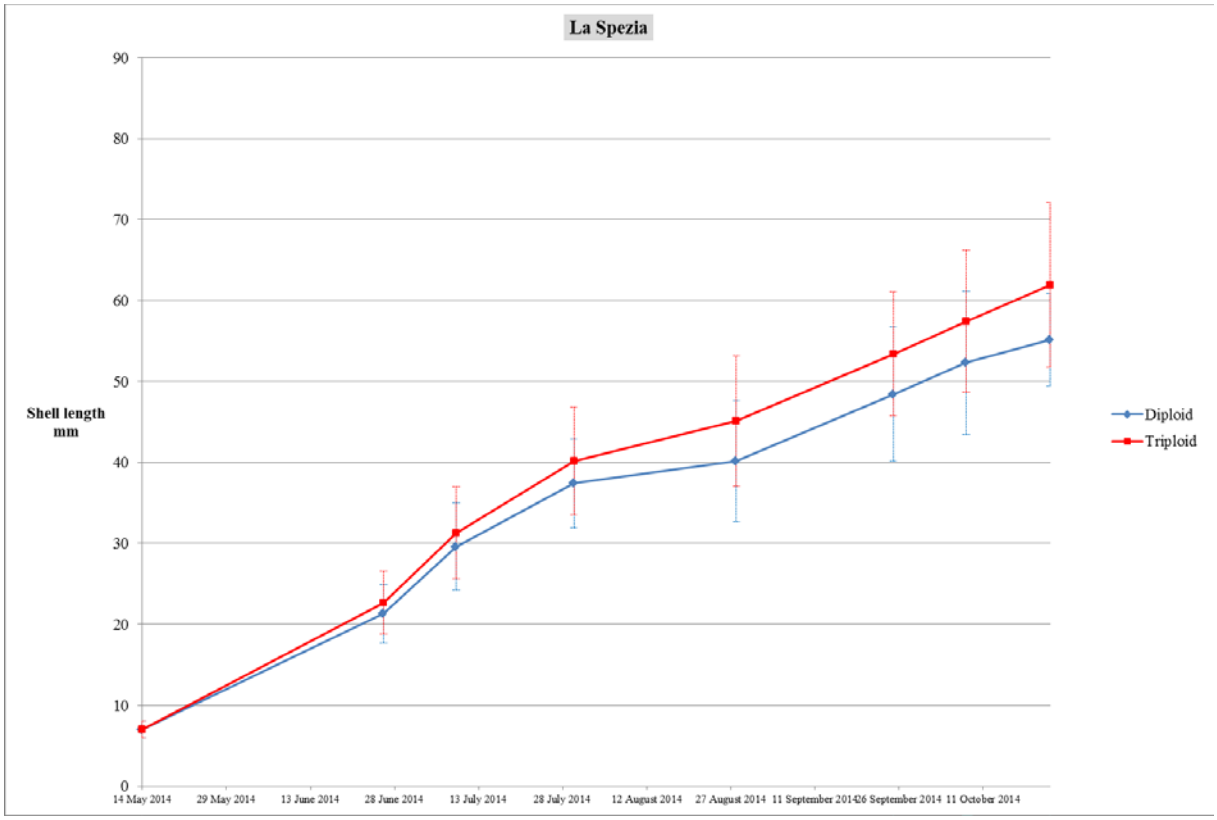


Fig. 48G Trend of the shell length of *C. gigas* individuals allocated in La Spezia in May 2014, expressed in mm, in function of time and ploidy.

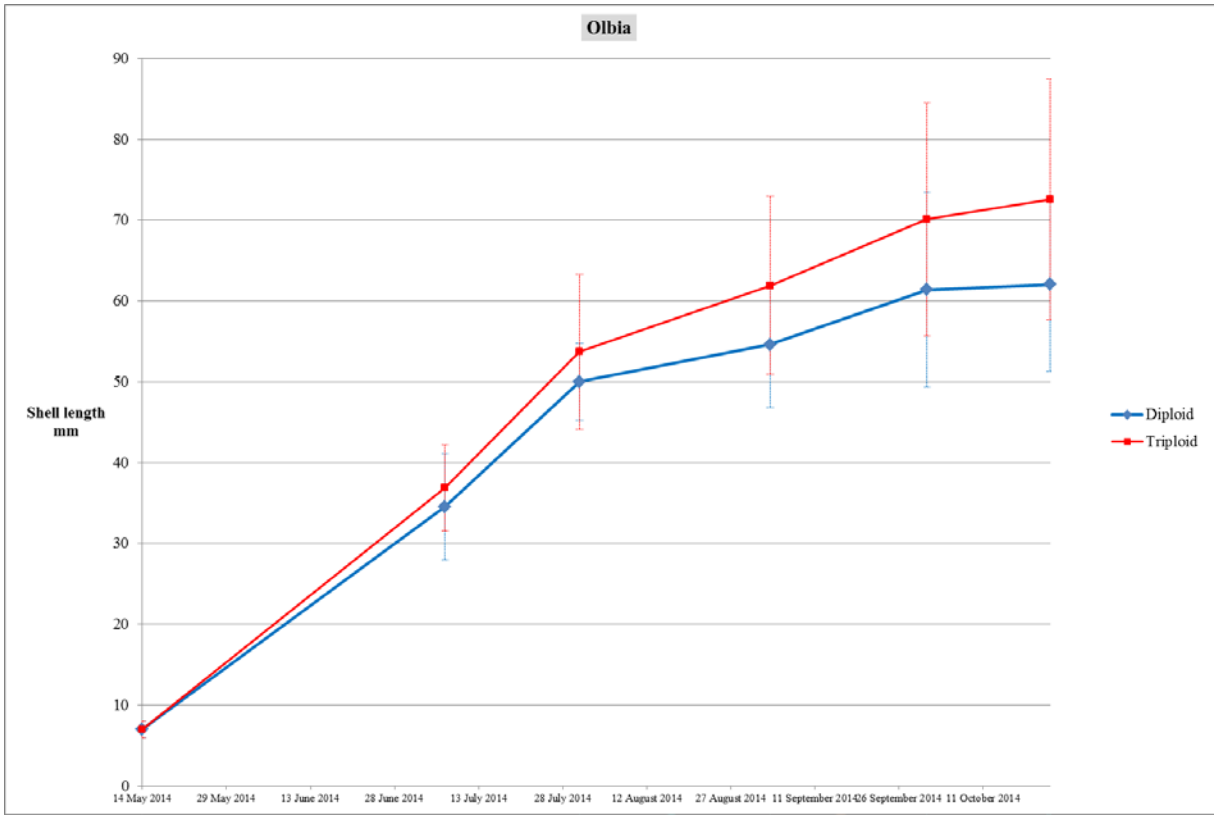


Fig. 48H Trend of the shell length of *C. gigas* individuals allocated in Olbia in May 2014, expressed in mm, in function of time and ploidy.

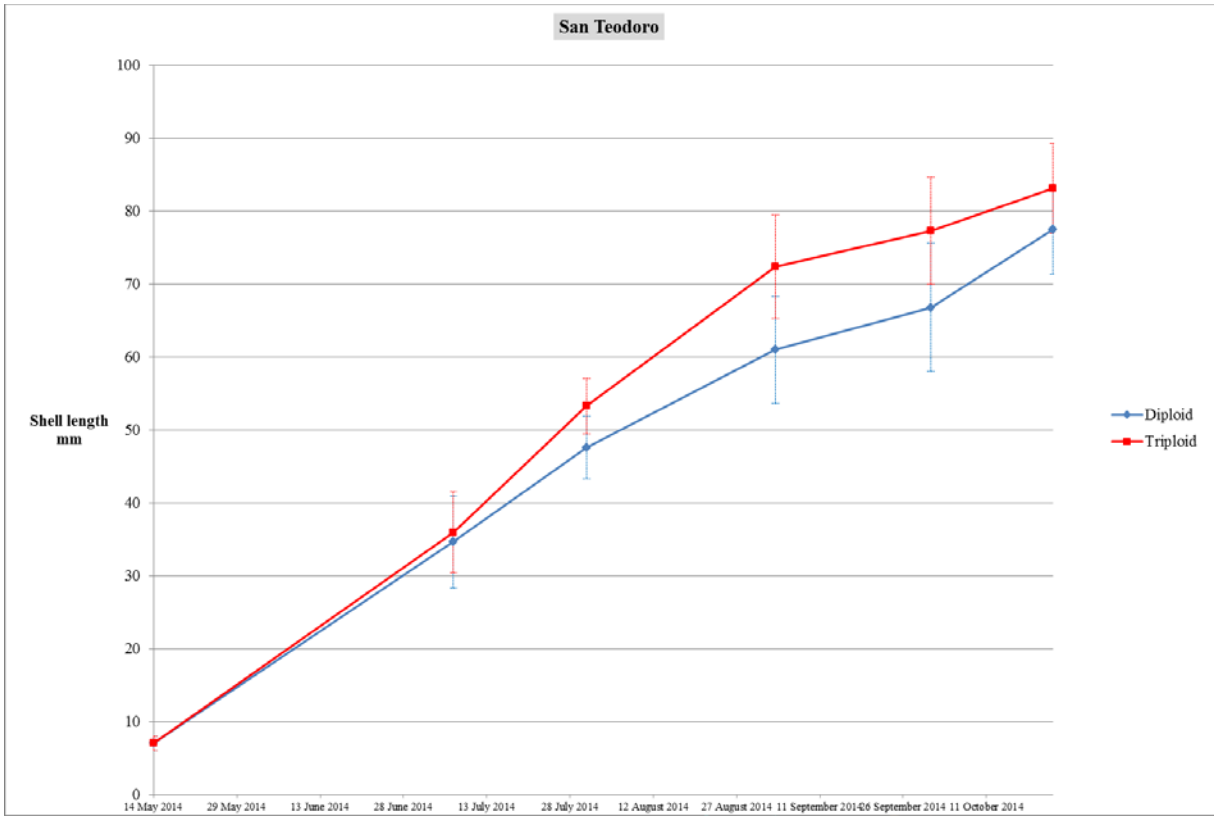


Fig. 48I Trend of the shell length of *C. gigas* individuals allocated in San Teodoro in May 2014, expressed in mm, in function of time and ploidy.

The growth was strongly related to the type of marine environment ($p<2e^{-16}$) for both triploid and diploid individuals. It resulted significantly higher in lagoons than in gulfs ($p<0.001$) and open sea ($p<0.001$). In fact, open waters appeared to be the less favourable conditions for oyster growth.

Within each type of environment, significant differences were observed in diploid individuals for gulfs ($p=0.00153$) and lagoons ($p=1.17e^{-8}$), while the two sites located in open waters gave similar performances. The site of La Spezia was characterised by a significant lower growth with respect to the other sites of the same environment type, which, on the contrary, did not differ from each other. San Teodoro showed significant better performances if compared to the other three lagoon sites ($p\leq0.0007$).

As noticed in diploid oysters, significant differences were observed in triploids in the sites located in gulfs ($p=0.003$), even if in a lesser extent than diploids since the growth in La Spezia resulted significantly lower only when compared to Olbia ($p=0.002$). Within lagoons ($p=0.0001$), the growth in San Teodoro was significantly faster only if compared to Orbetello ($p=0.0007$) while it was comparable in other sites. The growth in the two sites situated in open waters did not present difference in growth. The interaction between site and ploidy on

length was shown (p=0.022). As illustrated in Figure 49, the difference between triploid and diploid depends on the site and was lower in San Teodoro than Caleri even if these two sites are characterised by a rapid growth.

Fig. 49 Final shell length in mm in November 2014by ploidy and site.

Net obstruction

In the two sites located in open waters, Caorle and Giulinova, the net obstruction between two samplings was weak and represented mainly by mussel spat and algae of the Phylum Rhodophyta (Figure 50) and the maximum intensity was observed in June for both sites and year. The water flow within the lantern-net was never compromised.

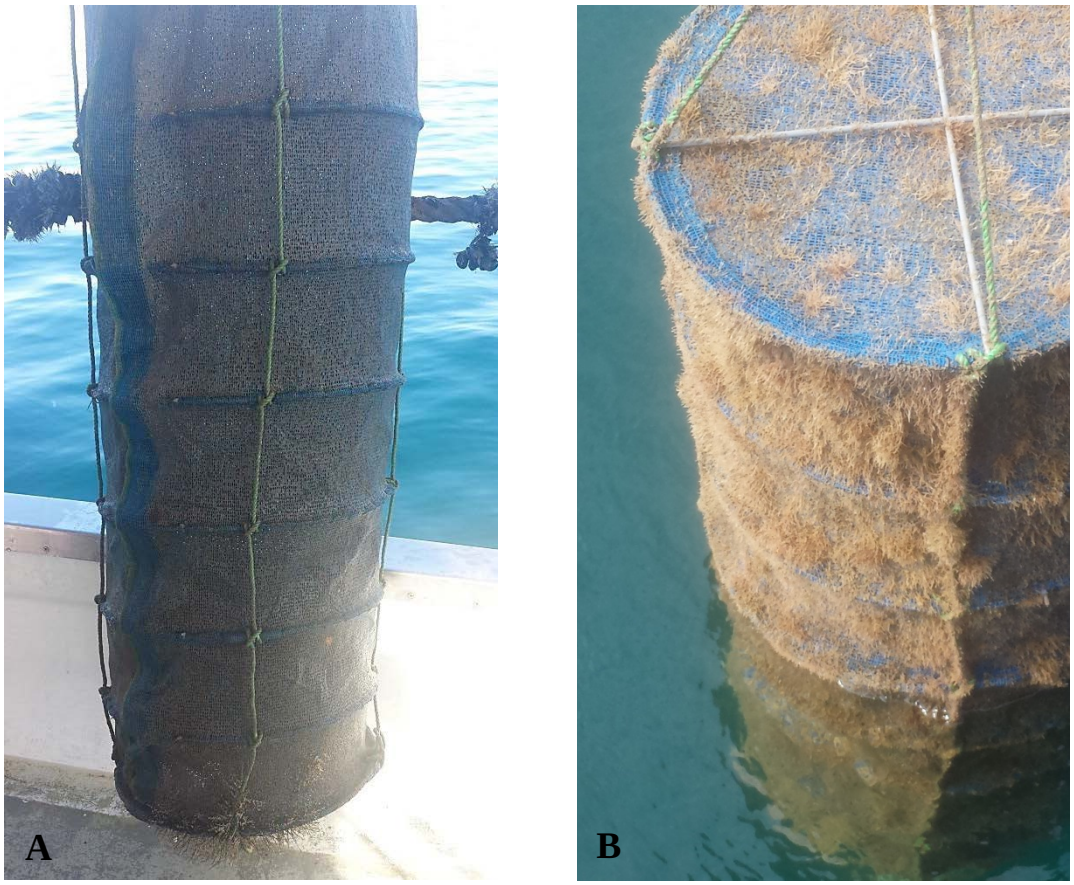


Fig. 50 Lantern-nets used in open water sites, in June 2014. A: Giulianova; B: Caorle.

The degree of obstruction in gulfs (La Spezia, Gaeta, and Olbia) was slightly higher if compared to open waters, but without impairment of the water flow through the lantern. The settlement of mussel spat was concentrated in all sites in June. During all the survey, we aslo noticed also the presence of ascidians, bryozoans, and sponges, with a peculiar panel of species for each site. On the contrary, in all the lagoons and during each sampling, except for San Teodoro where the farming technique reduces the settlement of marine organisms on floating bags, we observed a huge biofouling inducing the almost complete occlusion of the net. The identity of organisms settled was characterised by a cyclical pattern, but, in any case (site and season), the occlusion of the lantern was of very high intensity (Figure 51). For both years, in Caleri, Varano, and Orbetello, the net was noticed to be severely obstructed during each sampling despite regular washes or lantern substitution, and mud/ pseudofaeces were present in the compartments, especially in Caleri and Orbetello.

Fig. 51 Lantern-nets in lagoon sites, in 2014. A: Caleri (August 2014) ; B: Caleri (July 2015); C: Varano (August 2014).

The net present on the top and bottom of tubes used in France for the containment of T2 diploid spat during the first two/four months was still fairly open (Figure 52) but the device

did not allow an adequate water flow to prevent mud to accumulate inside the tube in a satisfactory way.

Fig. 52 Tube used in France in 2015 for the first months of allocation in farming site of the T2 diploid spat.

Environmental data

- *Temperature*

The trend of seawater temperature during the two years of the survey, at -0.5 m depth, is represented in Figure 53. We evidenced by red dotted lines the upper and lower threshold temperatures when mortalities associated with OsHV-1 usually occurred (Burge *et al*., 2007; Pernet *et al*., 2012; Petton *et al*., 2013; Jenkins *et al*., 2013; Paul-Pont *et al*., 2013b). In Italy, the permissive temperature for the occurrence of the viral disease is observed in Italy between middle April and early July, and between early September and November or December, depending on sites. In lagoon sites, water temperature fluctuations are very marked, due to the rapid effect of the variations of atmospheric temperature on shallow waters. Orbetello showed the widest range of temperatures with a minimum of 3.2 °C in January 2015 and a maximum of 32.1°C in July 2015. In France, the range of temperature comprised between16 and 25°C was recorded between early July and late October.

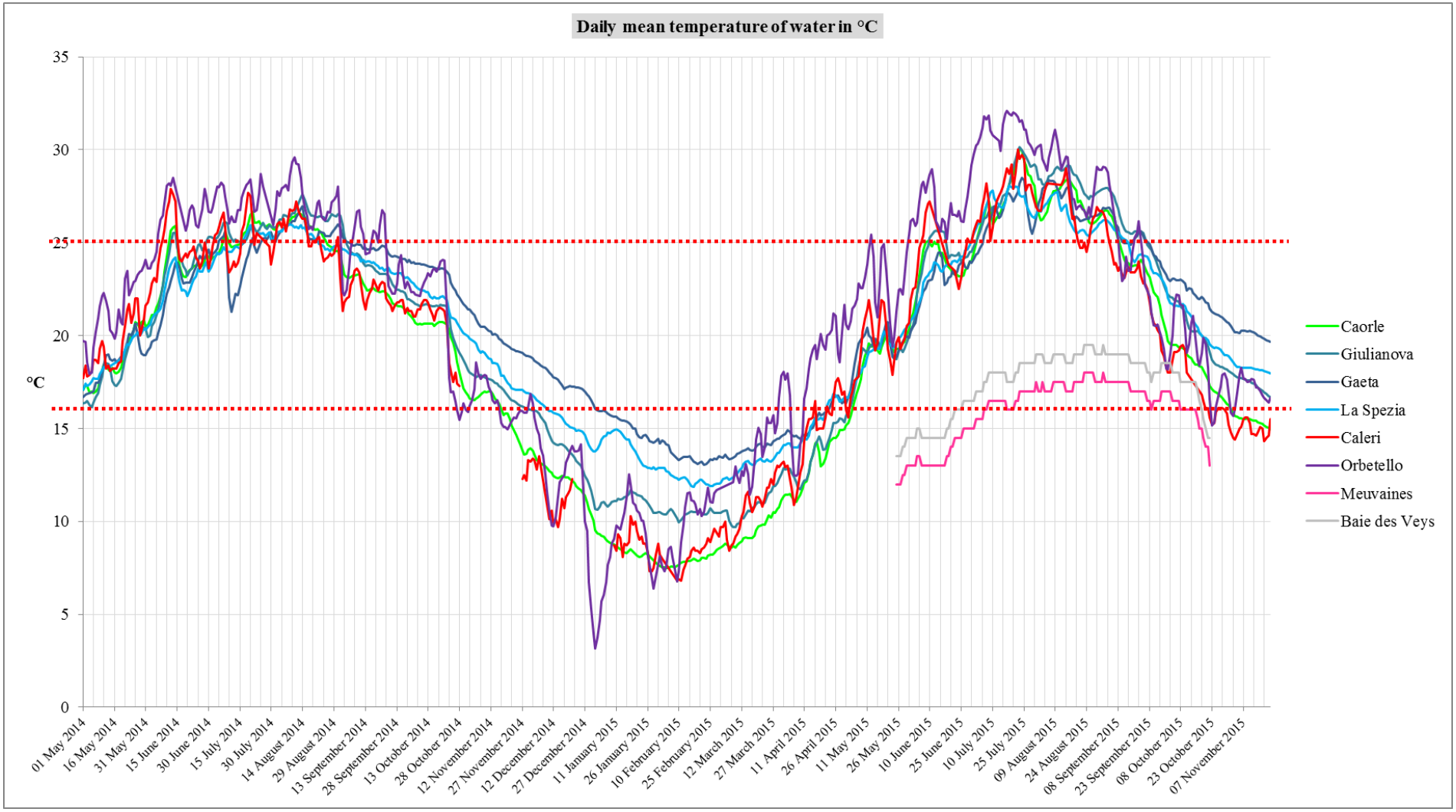


Fig.53 Graphical representation of the trend of water temperature recorded during the survey2014-2015. Red dottled lines represent the limits of temperature within which mortality was observed in other studies.

- *Salinity and anomalous rain events*

The evolution of water salinity during the two years of the survey, at -0.5 m depth, is represented in Figure 54. As observed for temperatures, important fluctuations of salinity were recorded for the sites located in lagoons and especially in Orbetello with a minimum salinity of 26.92 ‰ observed in late March 2015 and a maximum of 42.50‰ in early September 2014.

On the contrary, variations of salinity in open water, gulfs, and intertidal zones were moderate even if Caorle showed higher variability. In particular, a drop of water salinity was observed at the end of August 2014, following an anomalous rain event. Freshwater from rivers, also characterised by a high turbidity, was observed for one to two week as far as the Caorle farming site located 5 km from the coast line.

Unfortunately, no daily temperature and salinity data were available for Olbia, San Teodoro, and Varano, except for the measurements carried out during our samplings. In October 2014 and March 2015, also the sites of Olbia and San Teodoro, located in Sardinia, were affected by an anomalous rain event, which caused a dramatic drop of salinity until 6.9‰, and an increase of particulate in suspension.

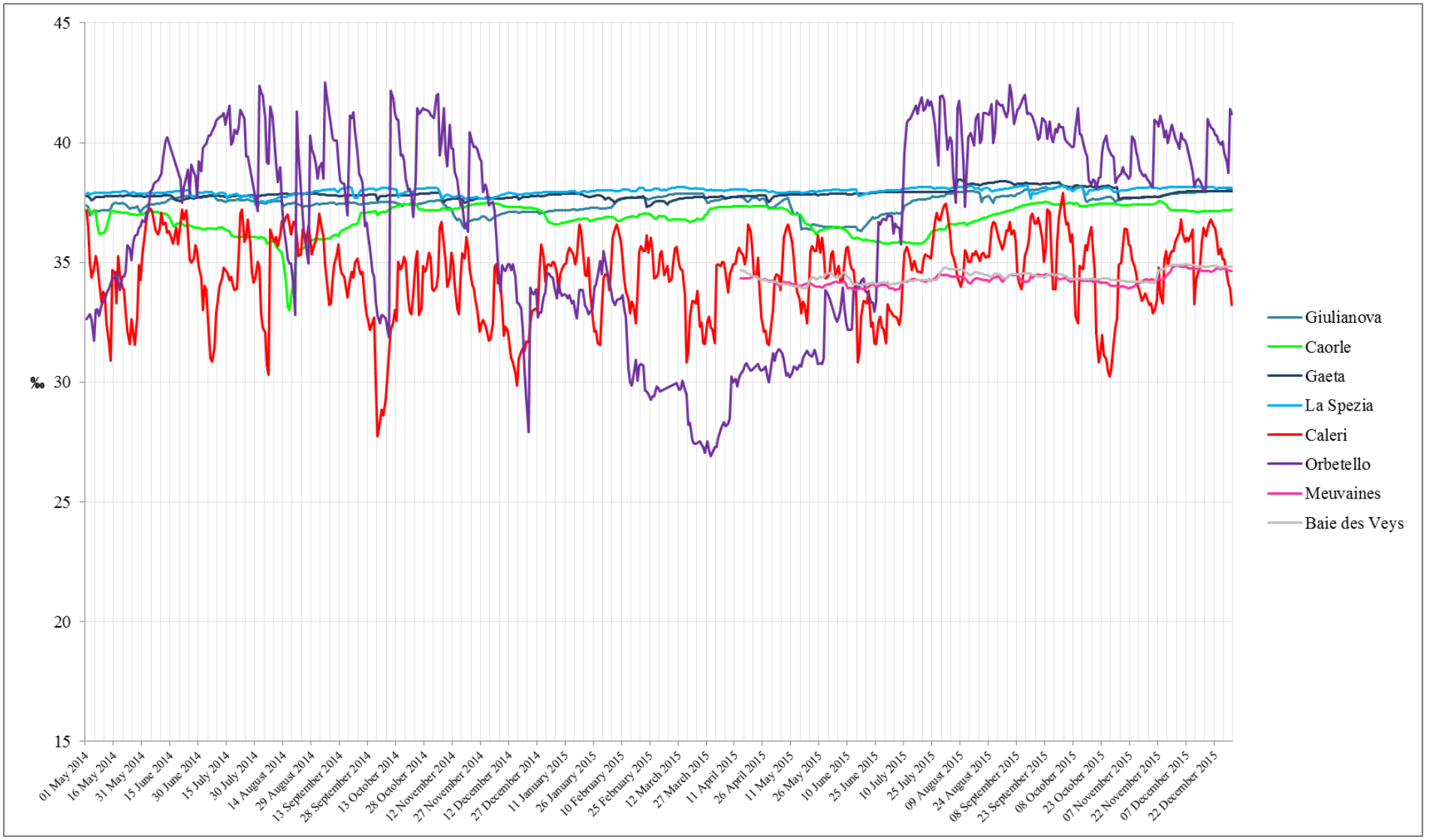


Fig.54 Graphical representation of the trend of water salinity recorded during the survey2014-2015

Mortality

For each allocation, about 5% of empty shells with an average length of 7 mm were found in each site at the first sampling and may be imputable to the stress of transport and acclimatisation of spat at its arrival in the new environment.

- *Cumulative mortality*

The highest cumulative mortality was observed in the site of Caleri, for both allocation dates (May and July 2014). Diploid and triploid individuals deployed in May showed 83% and 79% cumulative mortality respectively, while 100% was recorded in both diploids and triploids allocated in July. High cumulative mortality (>45%), concerning the individuals from the two allocation dates, was also observed in Caorle and Olbia. In Varano, only specimens from the "May" allocation (triploids and diploids) suffered a cumulative mortality >30%, while, on the contrary, in La Spezia batches allocated in June were mainly affected (>25%). In the site of San Teodoro a moderate mortality around 20% was observed in "June" batches. For all other sites, cumulative mortality was below 20%. The mortality in batches placed in September was recorded only for the two last samplings and in Varano and Caorle 16% and 80% mortality was observed respectively. In all other sites it was below 5%.

In Italy, for the year 2015, the individuals allocated in April in the site of La Spezia suffered the highest cumulative mortality rate for both ploidies (100%). Even if the site was affected again by mortalities in 2015, the situation observed in Caleri remained contained (~ 40%) if compared to 2014. The site of San Teodoro showed a cumulative mortality rate comparable to that reported in 2014 even if slightly higher. The periodic monitoring of mortality was not conducted in Giulianova, Varano, and Gaeta in 2015, but the cumulative mortality observed at the end of the experimentation was below 20 % in all sites and batches, except for triploids in Varano, whose mortality was 23%. It resulted particularly low in Orbetello with 8% mortality in diploids and 6% in triploids. In France, the two sites suffered high mortalities (>50% in all batches) and the site of Baie des Veys was mainly affected with 95% of cumulative mortality in the diploid batch, while it reached 74% in diploids in Meuvaines.

The monitoring conducted in 2015 on individuals placed in 2014 (>1 year-old) revealed a cumulative mortality ≥40% in San Teodoro and around 20% in La Spezia. In the two other sites (Caorle and Orbetello) it still lower than 8% and in Gaeta it reached 11% in triploids and 8% in diploids.

The trends of cumulative mortality are reported in Figure 55 (A to E). The sites of Giulianova, Varano, and Gaeta were not concerned by the graphical representation for 2015 since only two of three samplings were carried out during this year.

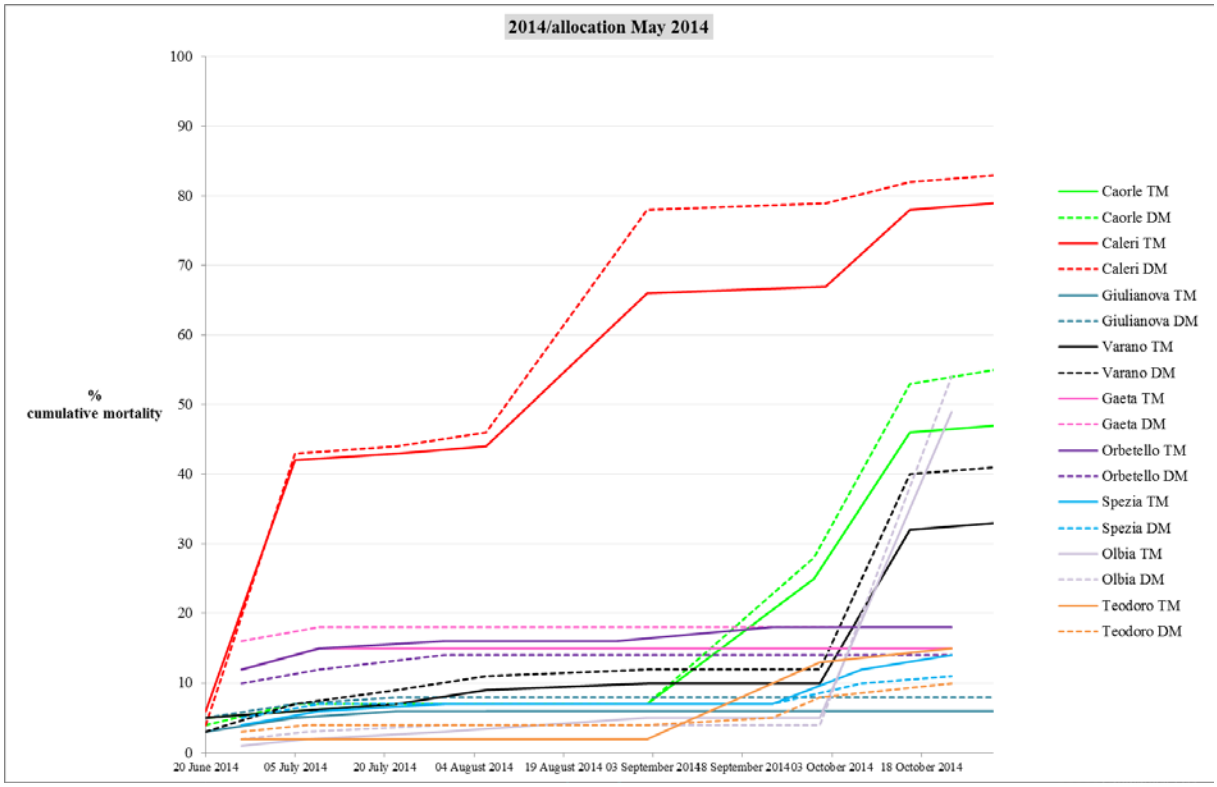


Fig. 55A Cumulative mortality observed in the different sites during 2014 in diploid and triploid batches allocated in May 2014.

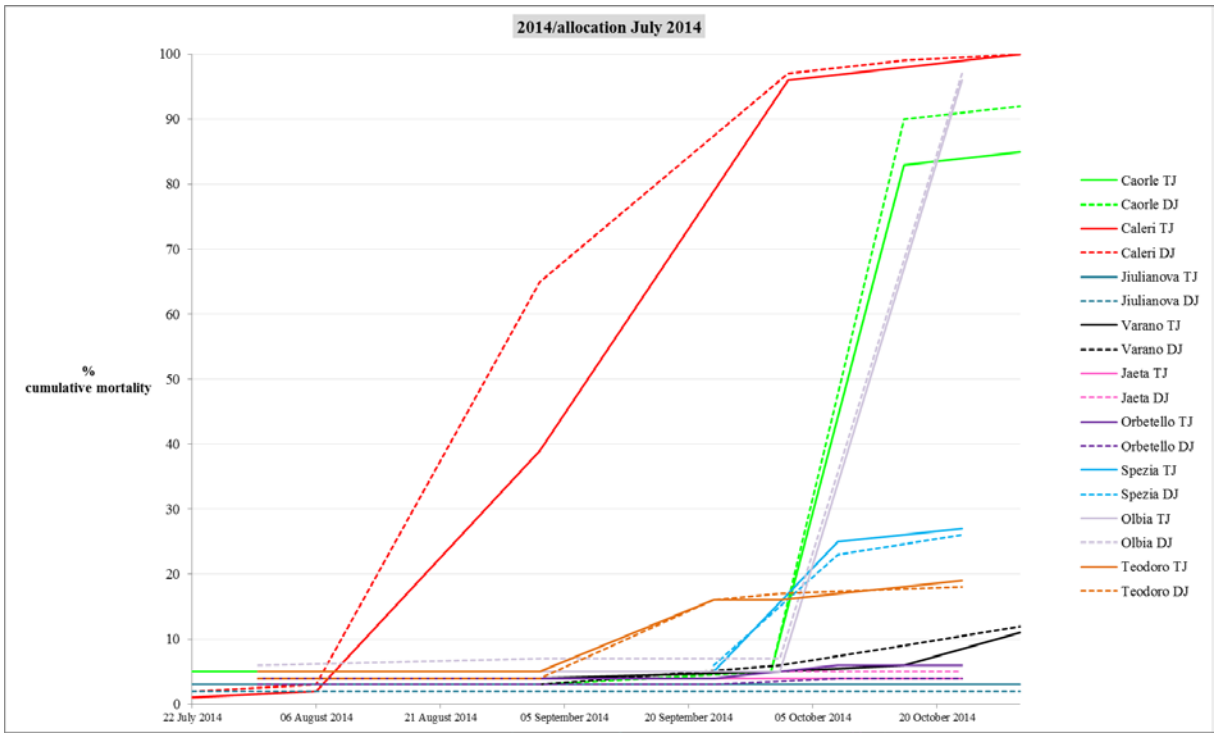


Fig. 55B Cumulative mortality observed in the different sites during 2014 in diploid and triploid batches allocated in July 2014.

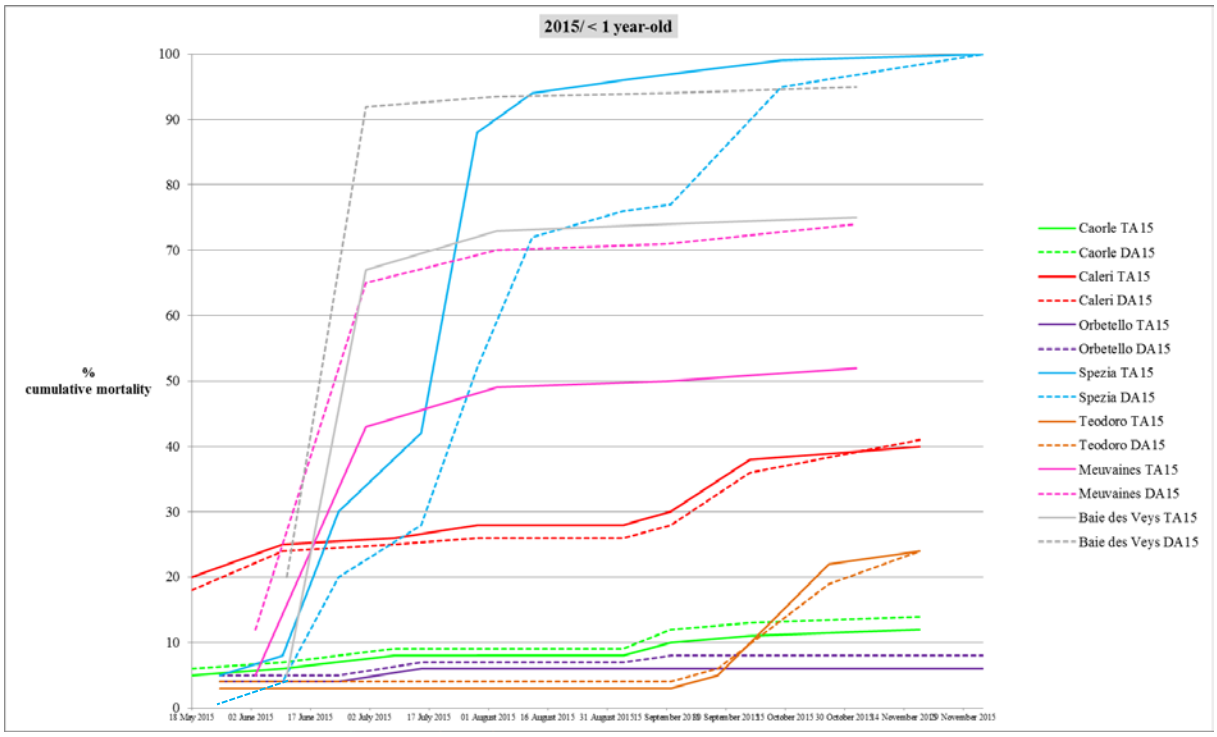


Fig. 55C Cumulative mortality observed in the different sites during 2015 in diploid and triploid batches allocated in April 2015.

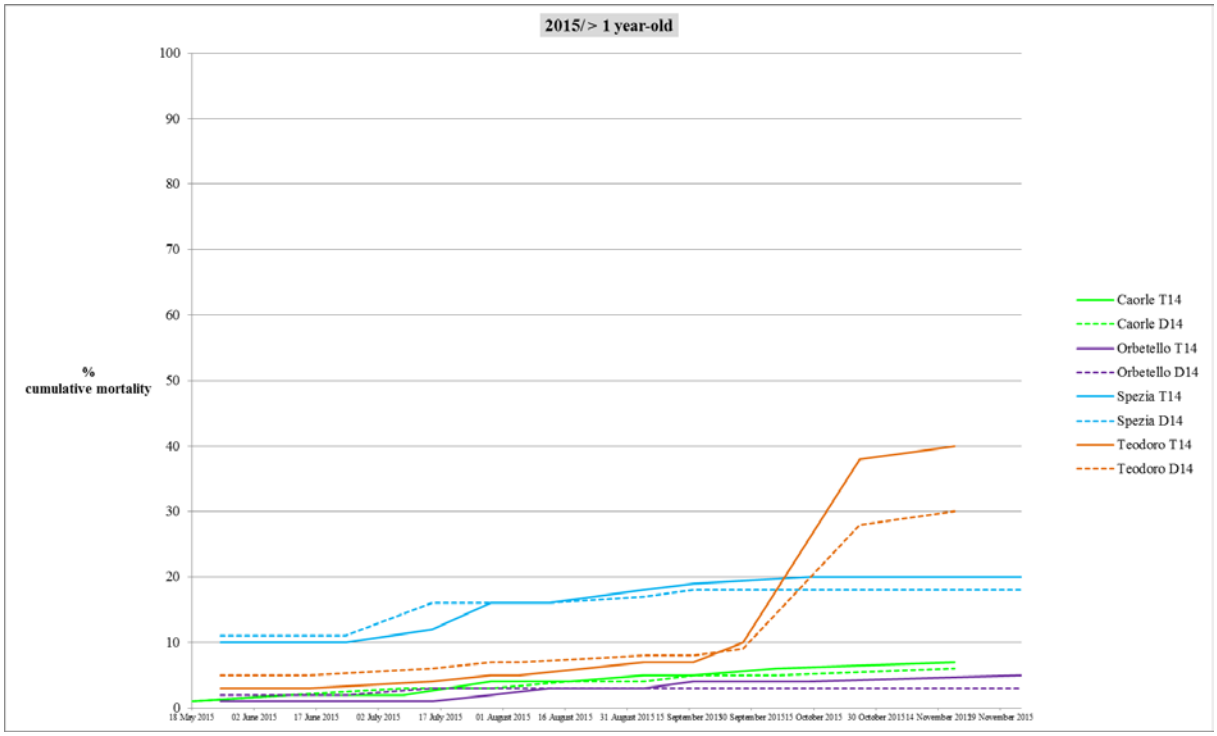


Fig. 55D Cumulative mortality observed in the different sites during 2015 in diploid and triploid batches allocated in 2014.

- *Allocation date, site, ploidy, and age on cumulative mortality rate*

In 2014, in the various sites were a cumulative mortality rate >20% occurred, the batches allocated in July suffered a significantly ($p<0.05$) higher mortality than those allocated in May, except for Varano, where the opposite trend was observed.

In 2014, as high cumulative mortality rates were recorded in some sites belonging to all the three different types of marine environment, then the site effect on mortality was excluded. On the contrary, in 2015, mortality in open waters was consistently lower ($p<0.05$) than in the lagoons and gulfs. In fact, if we compared the cumulative mortality rates in batches of individuals < one year-old (spat) in 2014 with the rates observed in 2015, the mortality was significantly lower in 2015 ($p<0.05$) in all sites except for La Spezia and, in a lesser extent, San Teodoro.

The two intertidal sites of Meuvaines and Baie des Veys suffered high mortality and conspicuous differences were observed between the batches on the basis of their ploidy, with significantly higher mortality in diploids for both sites ($p<0.05$). Unfortunately, in these sites, the survey was conducted only for the year 2015 so that no comparison between 2014 and 2015 was executable.

A significant difference in cumulative mortality was observed in spat among the three sites located in gulfs for both 2014 and 2015 with higher mortalities observed in Olbia (only for 2014) and La Spezia if compared to Gaeta ($p<0.05$), but the differences between the diploid and triploid individuals were never significant.

Differences in mortality rates were observed among the various lagoons, with significant higher mortality rates in Caleri ($p<0.05$), for both years. In this type of environment, differences between triploids and diploids were statistically significant, even if only for the batches from the allocation of May 2014 ($p<0.05$), with higher mortalities in diploids.

Interestingly, in the case of individuals > one year-old, the mortality was, on the contrary, higher in triploids than in diploids, in all sites. This difference was highly significant in San Teodoro ($p<0.05$).

- *Seasonality of mortality*

Between the allocation date in May 2014 and the sampling carried out in the third week of June, the individuals in the sites of Orbetello and Gaeta suffered a mortality ranging between 10 and 16%, depending on batches and sites, (Figure 56A). During the rest of the survey, theses batches were not affected by mortalities.

In the site of Caleri, particularly concerned by anomalous mortalities in 2014, the events affecting spat allocated in May were concentrated during the first half of July and a period comprised between the first half of September and the first half of October, with a peak at the beginning of September when the mortality between two samplings reached 60% in the triploid batch. Then, the phenomenon decreased gradually until it disappeared at the end of October. A similar evolution was observed in batches allocated in July 2014 (Figure 56B) even if the second half of September was the most pernicious. In 2015, the first peak of mortality, which occurred during the month of July in the previous year 2014, was not observed and the second peak, arisen in late summer/early autumn 2014, was weaker in 2015 (Figure 56C). The maximum mortality rate between two samplings in Caleri in 2015 was still ≤ 20% in all batches, throughout the survey, exceeding 10% only in May and in the first half of October. A peak of mortality was also observed in the site of Caorle between the end of September and the beginning of October 2014. The first half of October was problematic also for individuals placed in Varano and Olbia, which were affected by acute mortality events reaching 32% and 52% of mortality rate between two successive samplings respectively. In Olbia the batches allocated in July and in May suffered the mortality event simultaneously and, in Caorle, the phenomenon in batch allocated in July was more acute and began later. The study was not repeated in 2015 in Olbia, but in Caorle no anomalous mortality events were reported in 2015 (Figure 56C). A moderate peak of mortality, exceeding slightly 20% in triploids, was observed in La Spezia in the first half of October in batches allocated in July 2014. If, in general, mortalities in spat were lower in 2015 than in 2014, the site of La Spezia has been affected by anomalous high mortalities since June and during all the survey campaign in 2015. On the contrary, in the same period, individuals allocated in 2014 in this site (> one year-old) were not affected, even if the mortality in May was ≥10% (Figure 56E). Interestingly, all individuals farmed in La Spezia showed disturbances of the shell mineralisation, in fact shells appeared powdery and tenuous, with the presence of blisters containing colourless gelatinous material.

Among the other sites, only in San Teodoro the triploid individuals from both batches allocated in May and July suffered a mortality rate exceeding 10% in the second half of October 2014. Moreover, during winter 2014-2015 and in October 2015, individuals allocated in San Teodoro in 2014 (> one year-old) suffered a conspicuous mortality, with events reaching 30% of mortality rate and affecting mainly triploid specimens (Figure 56E).

In the two French sites, batches were subjected to mortality mainly in July (Figure 56D).

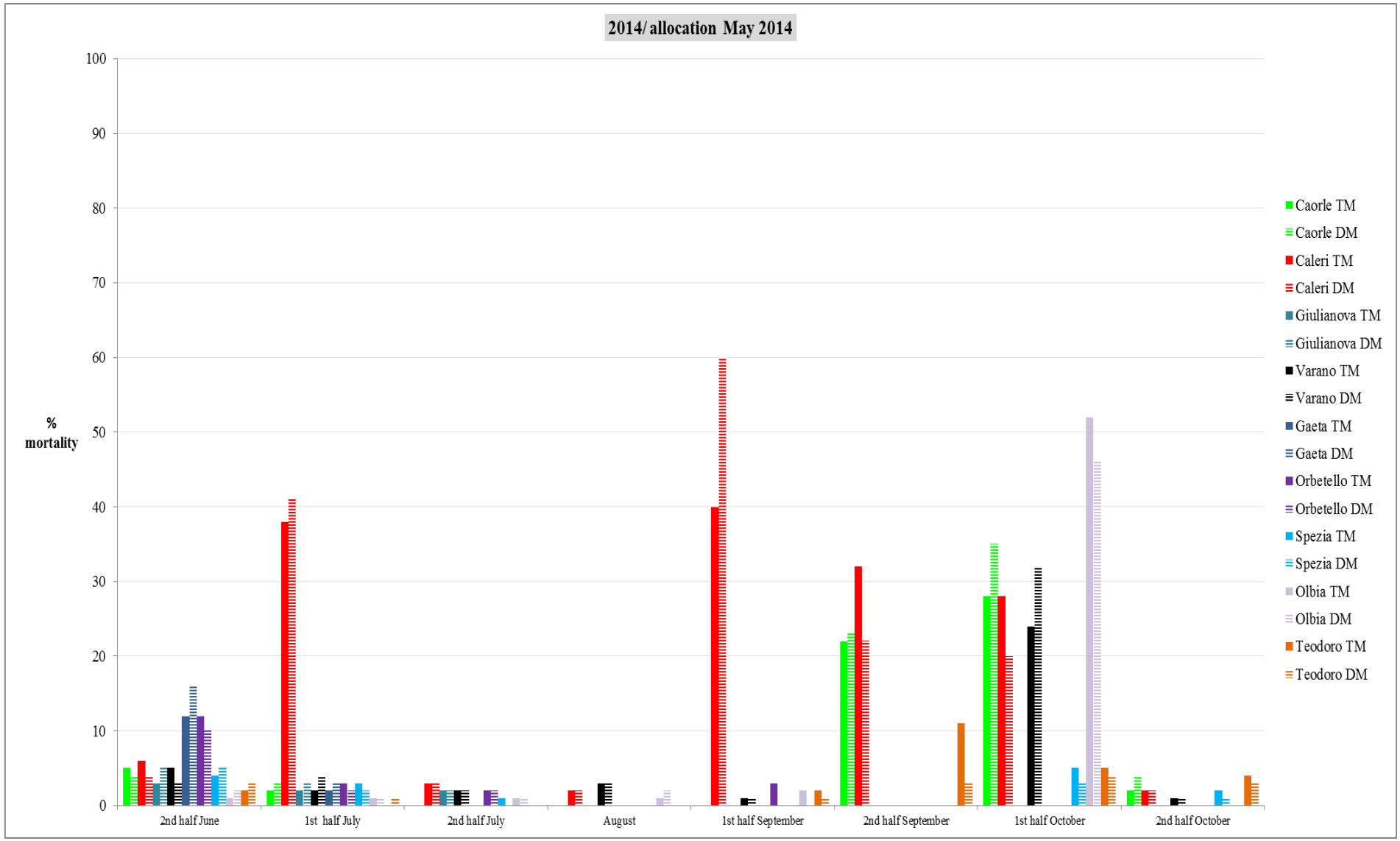


Fig. 56A Mortality rate between two successive samplings in 2014 in batches allocated in May 2014.

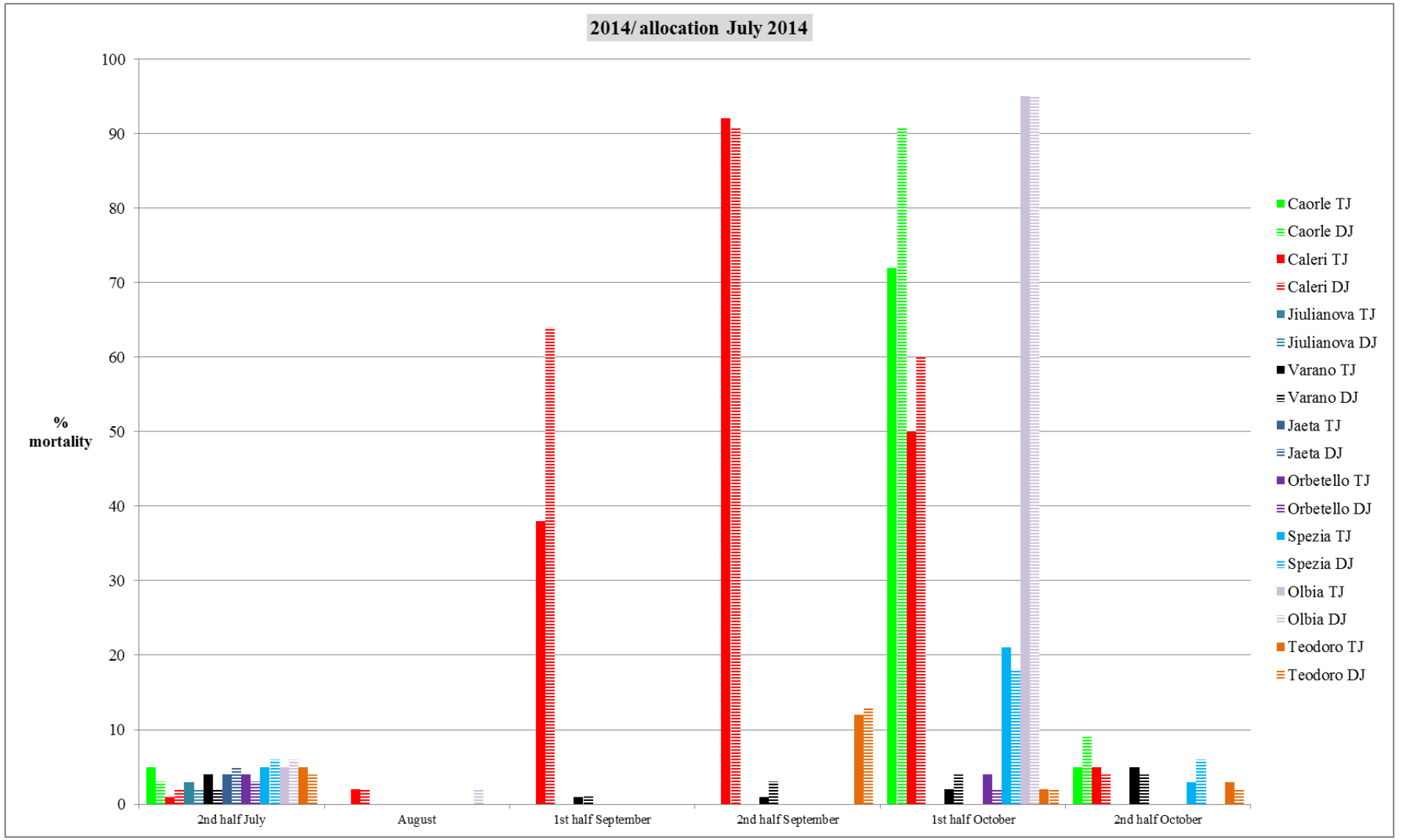


Fig. 56B Mortality rate between two successive samplings in 2014 in batches allocated in July 2014.

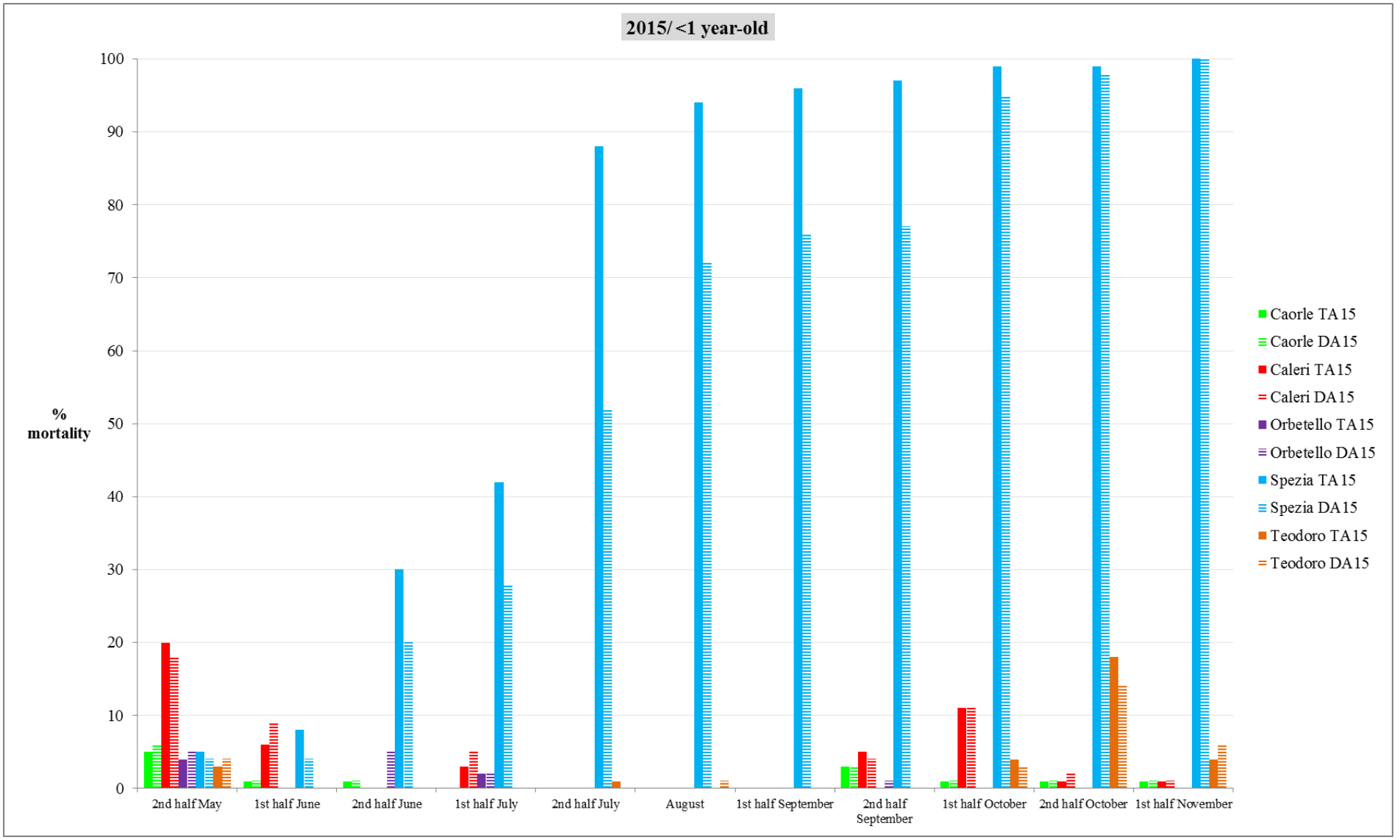


Fig. 56C Mortality rate between two successive samplings in 2015 in batches allocated in April 2015.

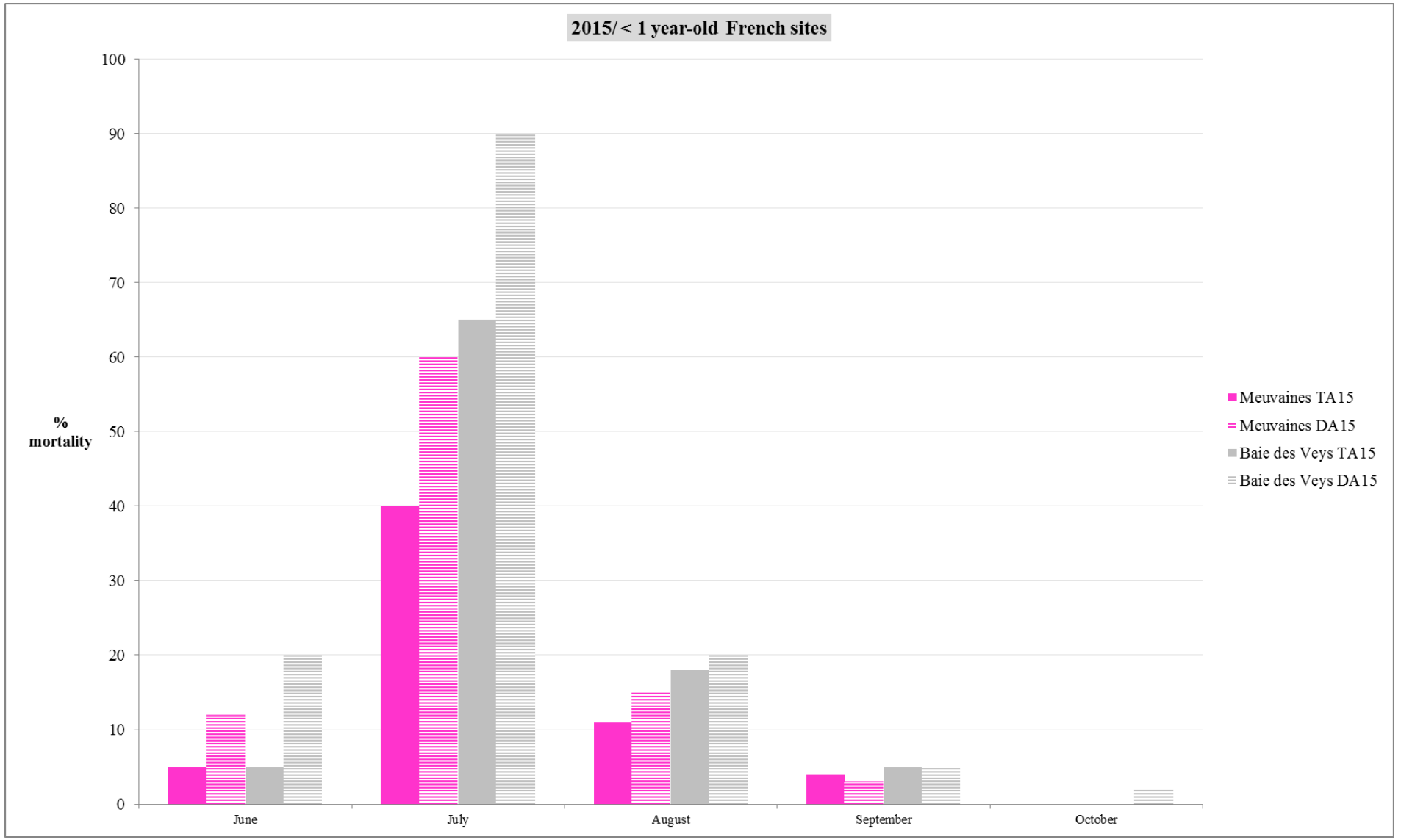


Fig. 56D Mortality rate between two successive samplings in 2015 in batches allocated in 2015 in France.

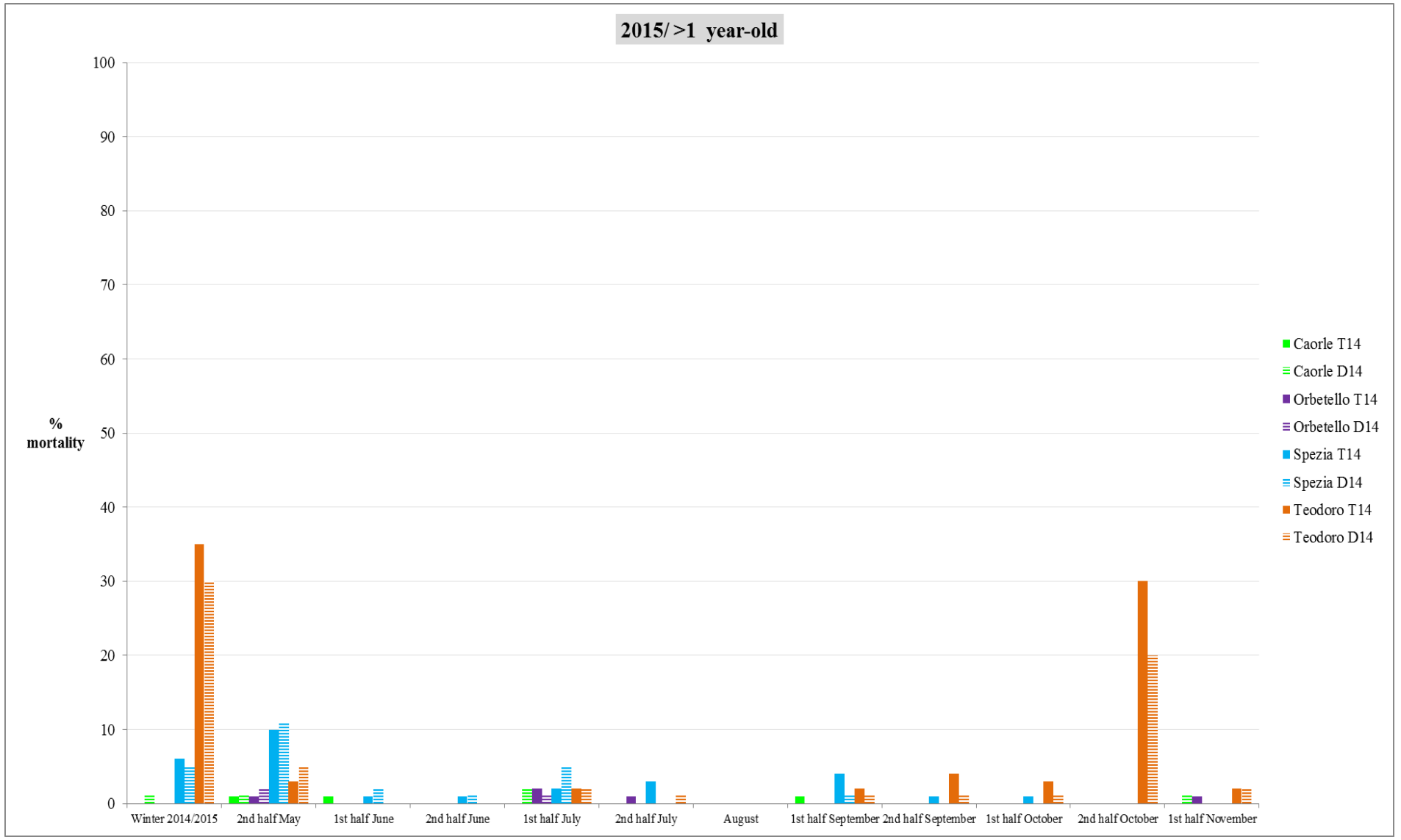


Fig. 56E Mortality rate between two successive samplings in 2015 in batches allocated in 2014.

OsHV-1 prevalence, load, and genotyping

- *Prevalence*

The presence of OsHV-1 in individuals placed in the sites of Caorle, Giulianova, and Gaeta was never detected during the present survey.

The maximum prevalence of OsHV-1 in spat in 2014 was observed in Caleri, during the first half of July, in the batch allocated in May (60%), and during the second half of September in the batch allocated in July (57%), showing a seasonal variation in the prevalence rate (Figure 57A, 57B). An increase of prevalence was simultaneously observed also in the batch of May (40%). In all other sites, the prevalence in spat was below 25%, but the presence of the virus was constantly detected throughout the survey in most sites (Figure 57A, 57B). No significant differences were observed between the maximum prevalence observed in individuals allocated in July and those allocated in May. However, in Caleri, the prevalence was significantly higher in both batches of triploid and diploid allocated in July if compared to those allocated in May.

The year 2015 was characterised by higher prevalence rates in spat, in all sites, if compared to 2014. In Italy, the maximum prevalence was observed in Caleri: 100% in the triploid batch during May 2015 (Figure 57C). With respect to 2014, the seasonality of the prevalence rate was more pronounced, with higher values in May and during the first half of June, except for La Spezia, where a peak of prevalence was observed during the first half of October (83% in the triploid batch). In most sites, we evidenced that after a drop of prevalence during the hottest months, prevalence turned to increase since September 2015. Prevalence of OsHV-1 in French sites reached 100% and 83 % in Baie des Veys and Meuvaines respectively, during July 2015(Figure 57D).

Even if comparisons are possible only for the sites of San Teodoro (represented in a separate graph Figure 57F because sampling was conducted also during winter), Orbetello, and La Spezia, the prevalence evidenced in 2015 was lower in older individuals if compared to spat in La Spezia and Orbetello, but resulted comparable in San Teodoro (Figure 57E, 57F). We observed that an increase in prevalence in May/June and autumn occurred in both Orbetello and San Teodoro sites, while, on the contrary, the peak of prevalence in La Spezia was observed only in autumn, later than in other two sites. These observations confirmed the evolution of prevalence reported in spat.

During all the survey, the prevalence was significantly higher in triploids (<0.05). However some exceptions were evidenced, such as in the sites of Meuvaines and Varano, where the prevalence was significantly higher in the diploid batches ($p<0.05$). In La Spezia,

during the year 2014, in the individuals from the allocation of July, OsHV-1 was detected only in the diploid batch, but if we consider all other batches issued from other allocation dates, the prevalence in triploid was significantly higher. In Orbetello, no significant differences in prevalence between diploid and triploid batches have been evidenced.

- *Viral load*

In 2014, viral loads exceeding the threshold value of 4.4 x 10^5 GU/50 mg, defined by Oden *et al.* (2011) as the limit above which the risk of mortality increases dramatically, were observed only in Caleri, in the triploid batches deployed in May, during the first half of July when a maximum viral load of 3.4 x 10^7 GU/50 mg was reached, and during the second half of September (1.4 x 10^6 GU/50 mg), and in La Spezia, during the second half of October (5.5 x 10^5 GU/50 mg) (Figure 58A). In batches allocated in July, only in Caleri, during the first half of October 2014, the triploid batch exceeded slightly the limit load (5.5 x 10^5 GU/50 mg) (Figure 58B). On the contrary, high viral loads were observed in Caleri and San Teodoro during the last sampling of the survey 2014, during the second half of October, in the batch of triploid spat allocated in September 2014. Even if in the main part of the sites, OsHV-1 was not diagnosed in this batch deployed in September, in Caleri the prevalence was 60% with a maximum load of 2x10^7 GU/50 mg of tissue, and in San Teodoro 50% prevalence was observed and the viral load reached 5.7x10^6 GU/50 mg. We did not represent graphically this data because only two sampling were carried out on this batch. However, in early spring 2015, during a check conducted on each site to prepare the allocation campaign 2015, the individuals from the batch characterised by the detection of a high viral load in autumn 2014 in Caleri and San Teodoro, appeared to not have been affected by mortalities during autumn and winter.

In 2015, the viral loads were generally comparable with what observed in 2014 (Figure 58C). Exceedings of the limit value were detected only in the site of Caleri, during May, in both triploid (1.3 x 10^6 GU/50 mg) and diploid (5.5 x 10^5 GU/50mg) batches, during early June in triploid (1.2 x 10^6 GU/50 mg), and during the second half of September in triploid (3.5 x 10^6 GU/50 mg), and in the site of San Teodoro, in the triploid batch, during the first half of June (7.5 x 10^5 GU/50 mg). As the allocation campaign was anticipated to April instead of May in 2015, we observed that a peak of viral load occurred in these two sites between May and June. As for prevalence, the peak of viral load observed in Caleri in July 2014 was not repeated in 2015. In France, in both sites, the threshold was crossed during July

but only for the diploid batch in Meuvaines (5 x 10^8 GU/50 mg) and only for the triploid batch in Baie des Veys (2.4 x 10^7 GU/50 mg) (Figure 58D).

The viral loads observed in 2015 in individuals allocated in 2014 never exceeded the limit load of 4.4 x 10^5 GU/50 mg being lower than 2.10^4 GU/50 mg in all sites (Figure 58E) except for San Teodoro, where an increase in the viral load was observed essentially in December 2014 (7.4 x 10^4 GU/50 mg), March 2015 (3.2 x 10^4 GU/50 mg), and May 2015 (6.7 x 10^4 GU/50 mg) (Figure 58F).

- *Genotyping*

When positivity to OsHV-1 was evidenced by real-time PCR in a sampled batch, from one to two extracted of DNA of positive individuals were analysed by conventional PCR followed by sequencing. Fiftyseven samples collected in 2014, characterised by a sufficient viral load to be sequenced (a C_T value of 35.5 were used as upper limit), were subjected to genetic analysis. Twenty-nine sequences of the C2/C6 region were obtained, and only 25 and 24 the ORFs 42/43 and ORFs35-38 respectively. For the year 2015, 83 samples were selected and we obtained 62 sequences of the C2/C6 region, 58 of the ORFs 42/43, and 59 of the ORFs 35-38. In Table 11, we reported the detail of the samples that were successfully sequenced per site.

	C2/C6		**ORFs 42/43**		**ORFs 35-38**	
	number of sequences		number of sequences		number of sequences	
	2014	*2015*	*2014*	*2015*	*2014*	*2015*
Caleri	8	10	7	9	7	9
Varano	5	1	2	1	1	1
Orbetello	3	4	3	3	3	4
La Spezia	5	7	5	8	4	8
San Teodoro	6	30	6	27	6	27
Olbia	2	-	2	-	2	-
Meuvaines	-	5	-	5	-	5
Baie des Veys	-	5	-	5	-	5

Table 11 Number of sequences obtained from the different regions targeted for the sequencing study, by site.

All the virus specimens, found to infect oysters in the different sites during the present survey, belonged to the genotype µVar as it was defined by Segarra *et al*. (2010), except a single specimen, found in La Spezia in 2015, in which one of the two single nucleotide mutations in the ORFs42/43 region, characteristic of the µVar genotype, was absent while

three substitutions were detected in this region, no amplification of the C2/C6 was obtained, and the deletion in ORFs 35-38 was absent, being more similar to the reference genotype even if eight nucleotide differences were present (Appendix D.3).

Even if all other specimens may be defined as µVar, several mutations were observed in the three regions as reported in Appendix D.2 and D.3, especially in specimens from the sites of Caleri and San Teodoro. Interestingly, none of the genotypes observed in wild specimens was found in the individuals deployed for the present study, except for Variant C and Variant E, whose presence was highlighted in San Teodoro in 2015 several times in some triploid individuals > one year-old, during and outside mortality events.

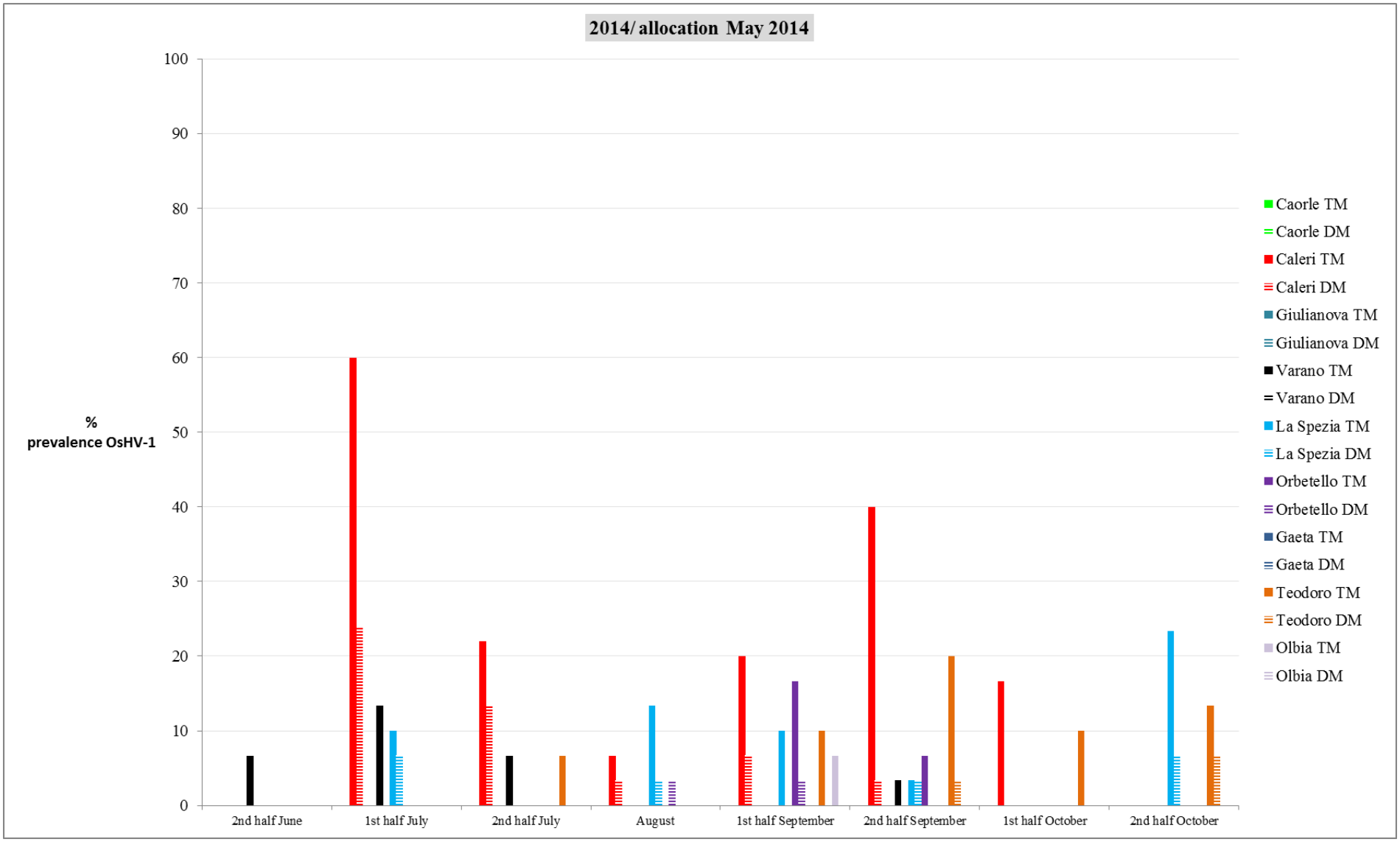


Fig.57A Prevalence of OsHV-1in Italian sites in 2014 in spat allocated in May.

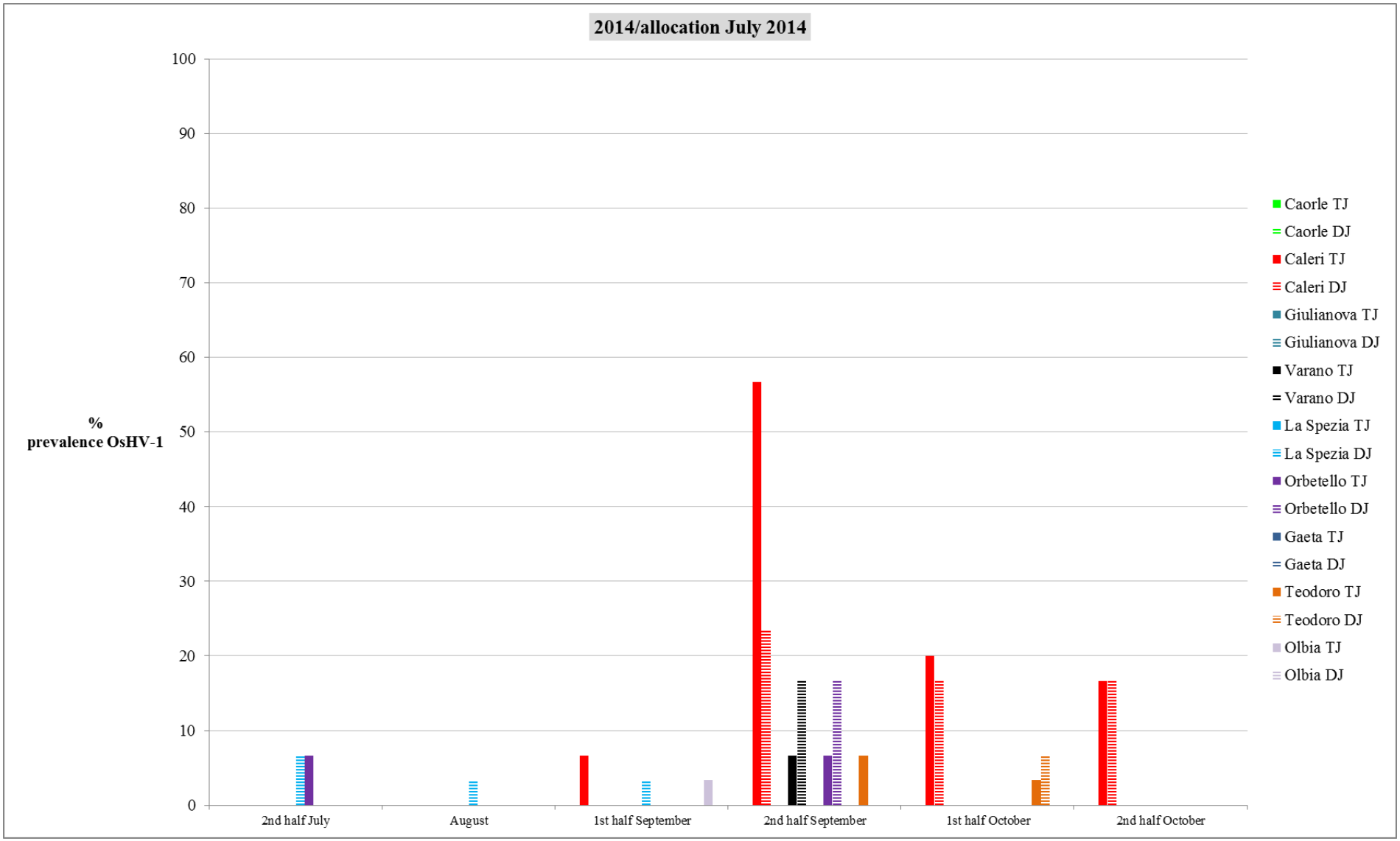


Fig.57B Prevalence of OsHV-1in Italian sites in 2014 in spat allocated in April.

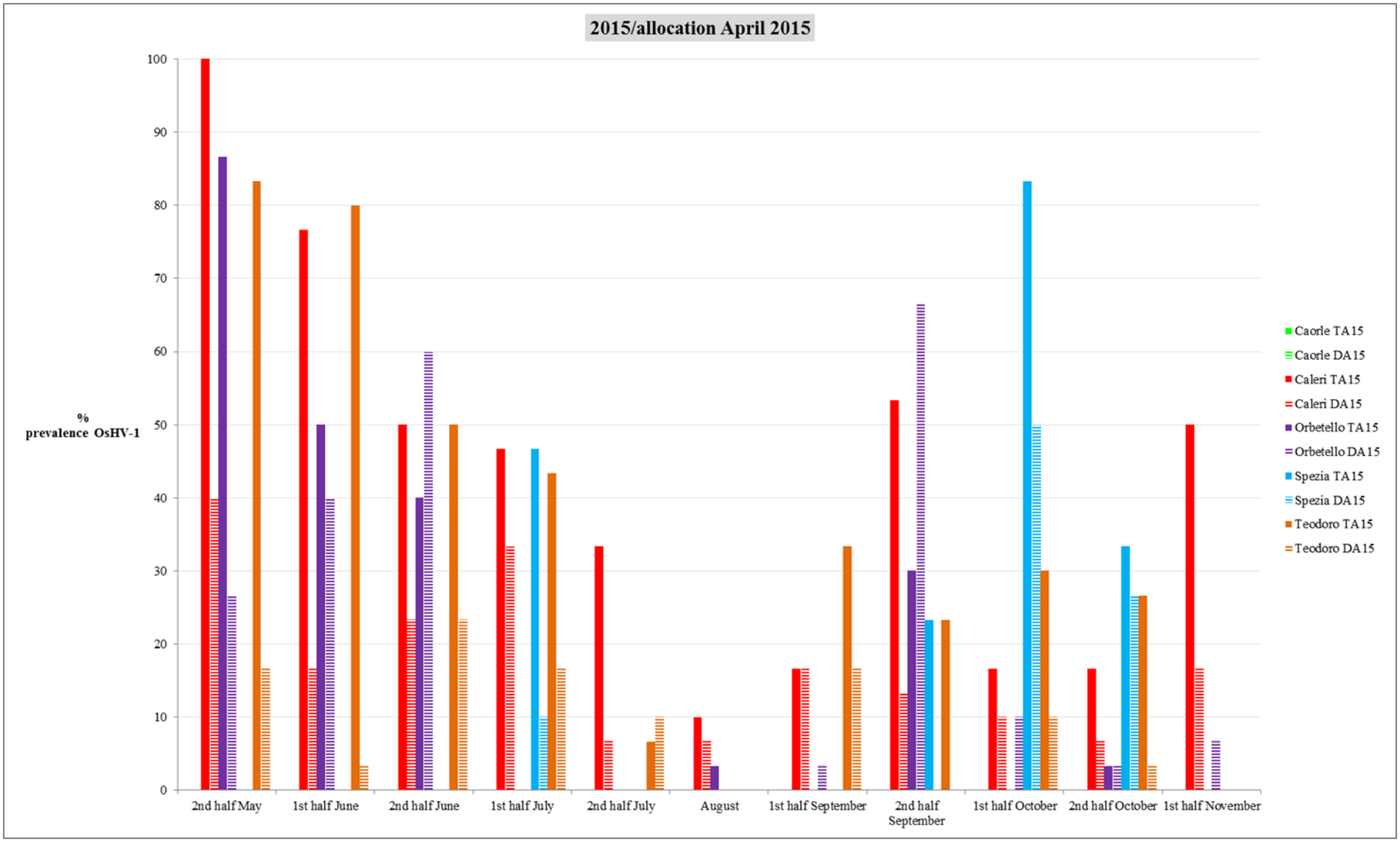


Fig.57C Prevalence of OsHV-1in Italian sites in 2015 in spat allocated in April 2015.

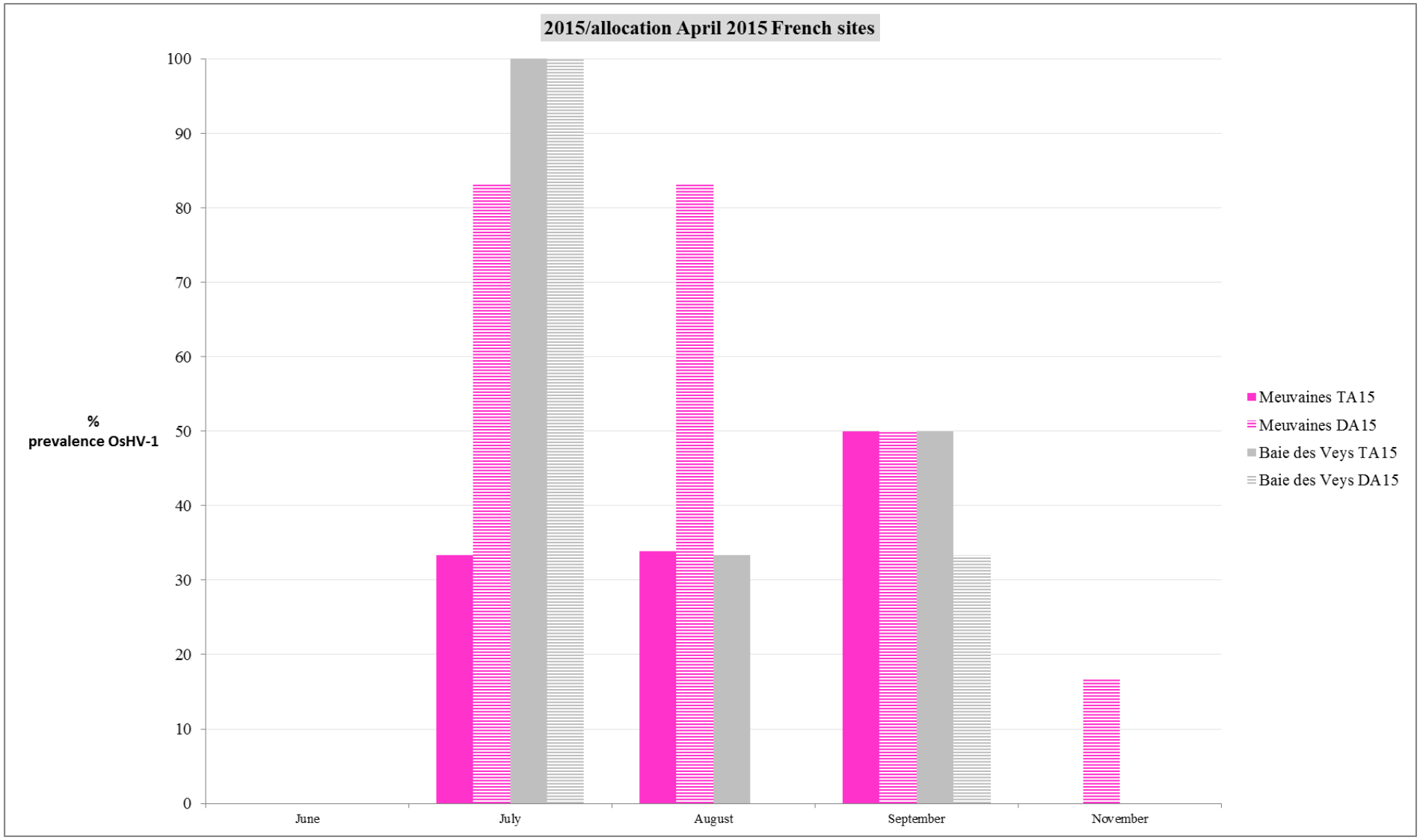


Fig.57D Prevalence of OsHV-1 in French sites in 2015 in spat allocated in April 2015..

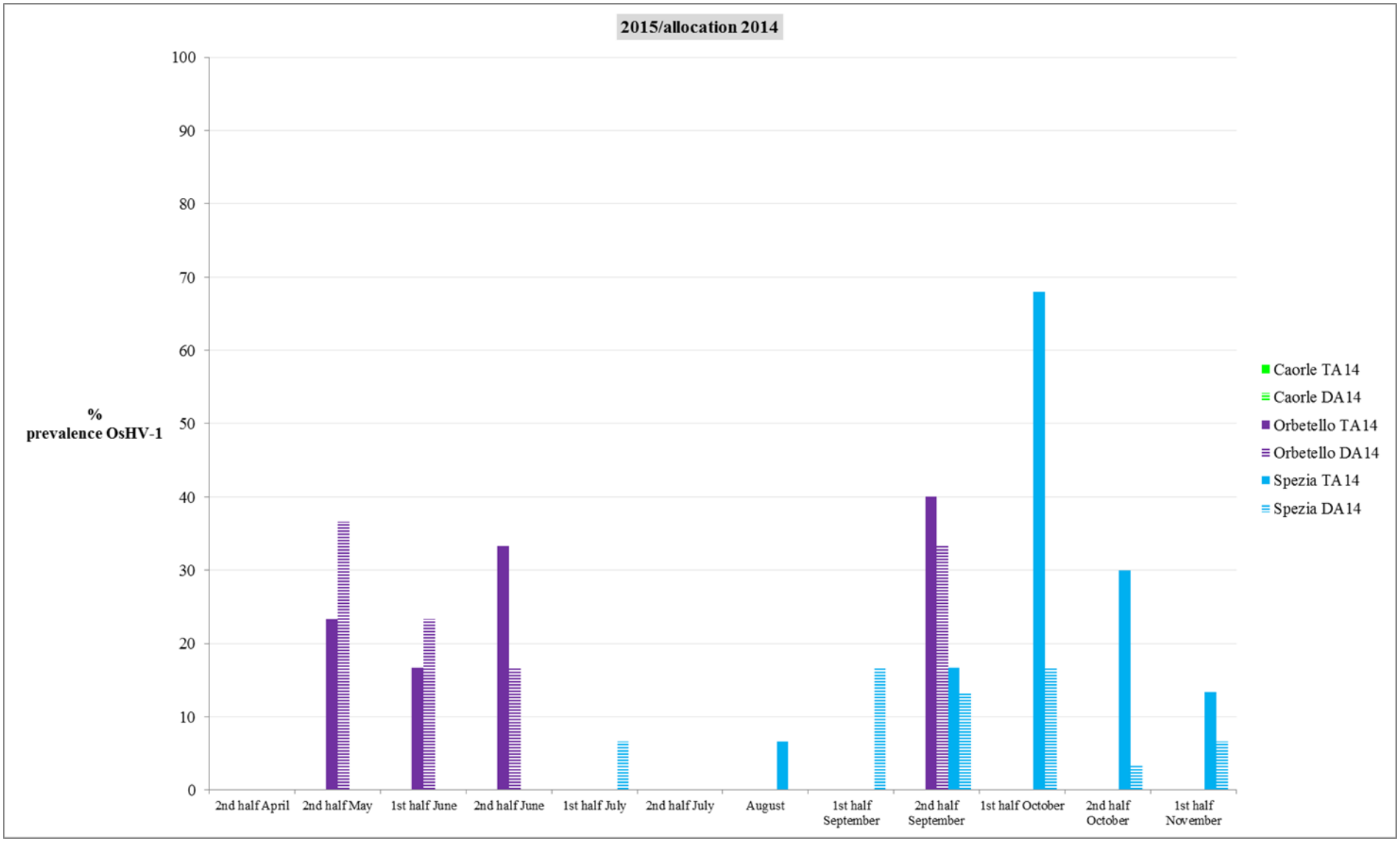


Fig.57E Prevalence of OsHV-1 in Italian sites in 2015 in spat allocated in 2014.

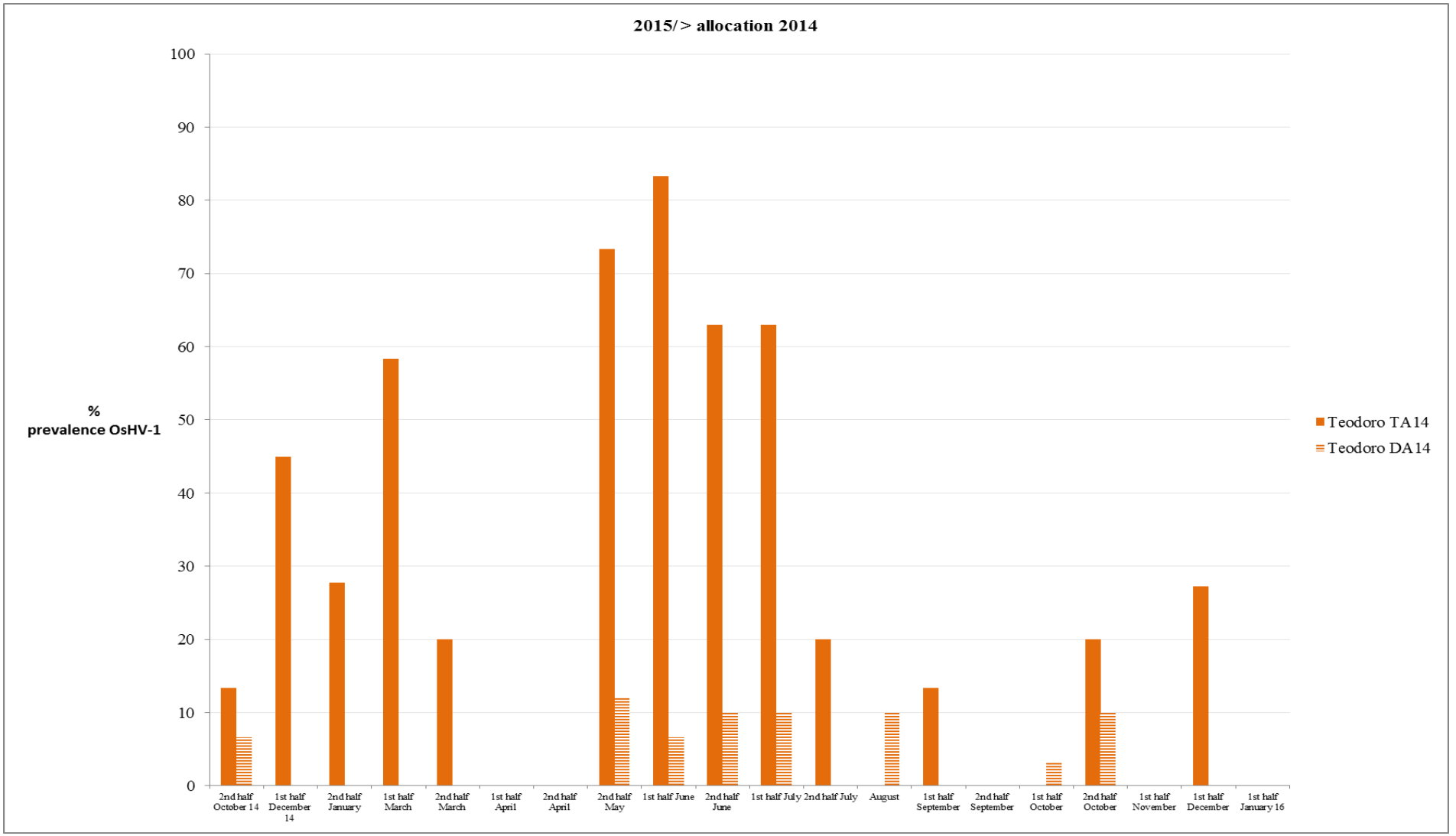


Fig.57F Prevalence of OsHV-1 in San Teodoro in 2015 in spat allocated in 2014.

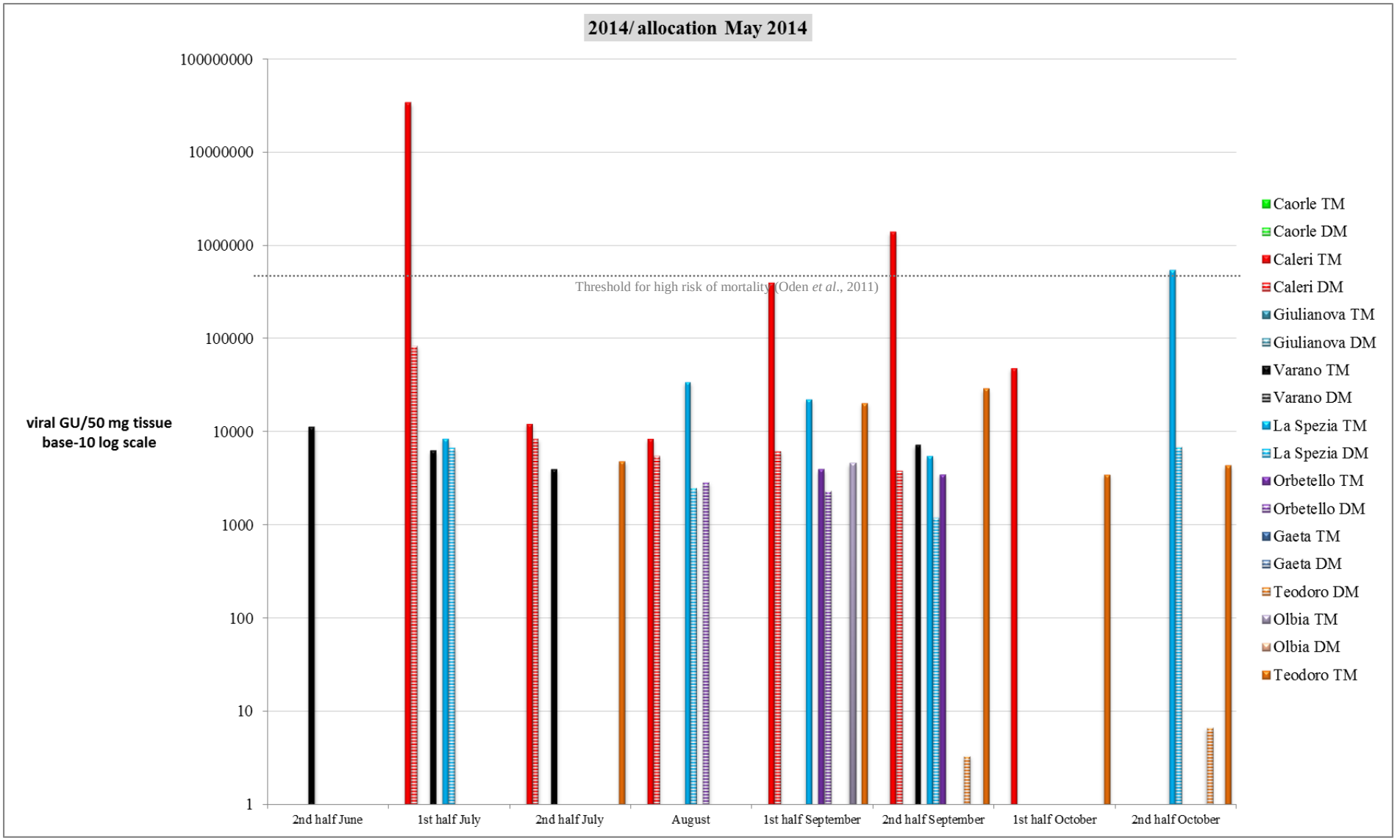


Fig.58A Mean viral load of OsHV-1in positive samples in Italian sites in 2014 in spat allocated in May.

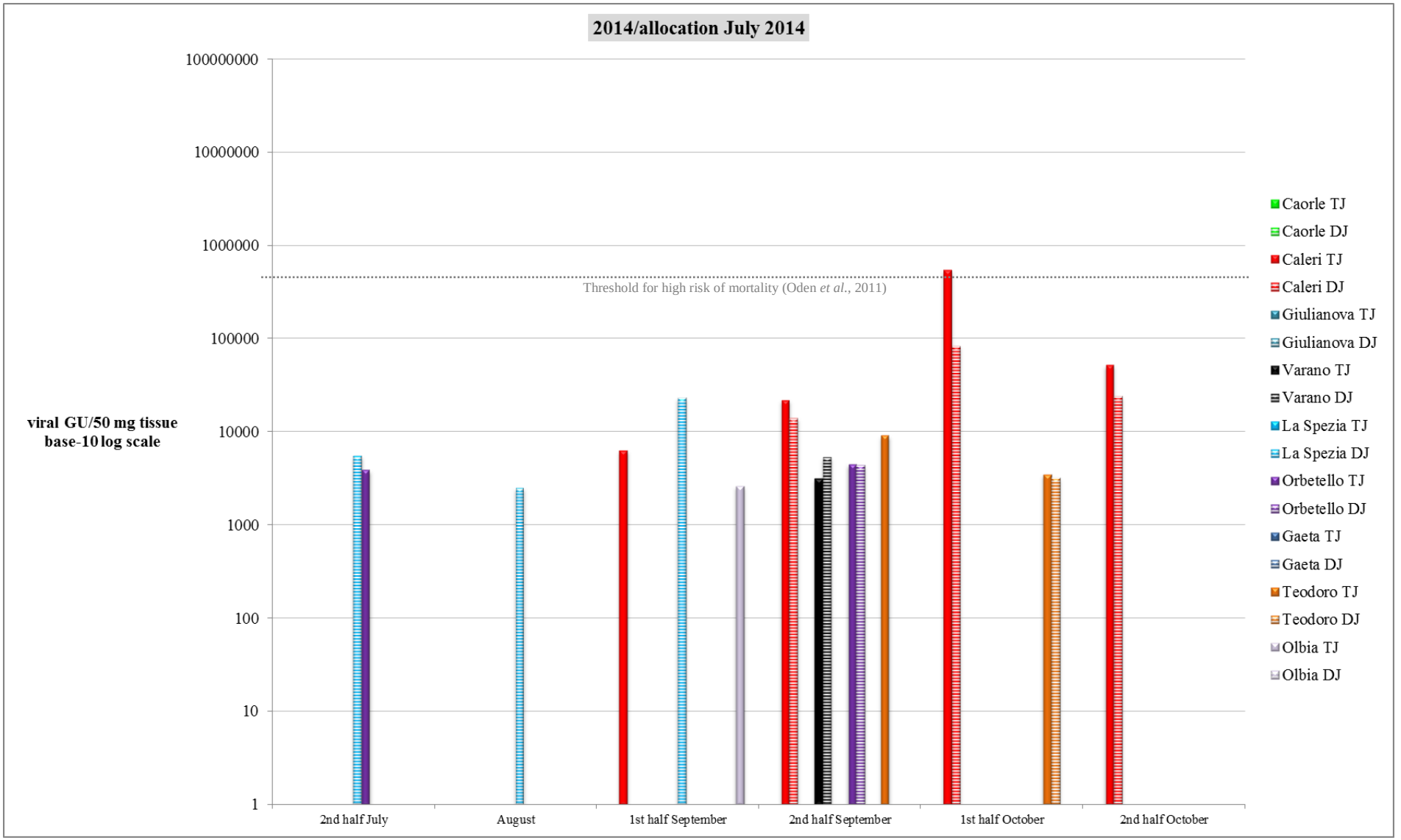


Fig.58B Mean viral load viral load of OsHV-1 in positive samples in Italian sites in 2014 in spat allocated in July.

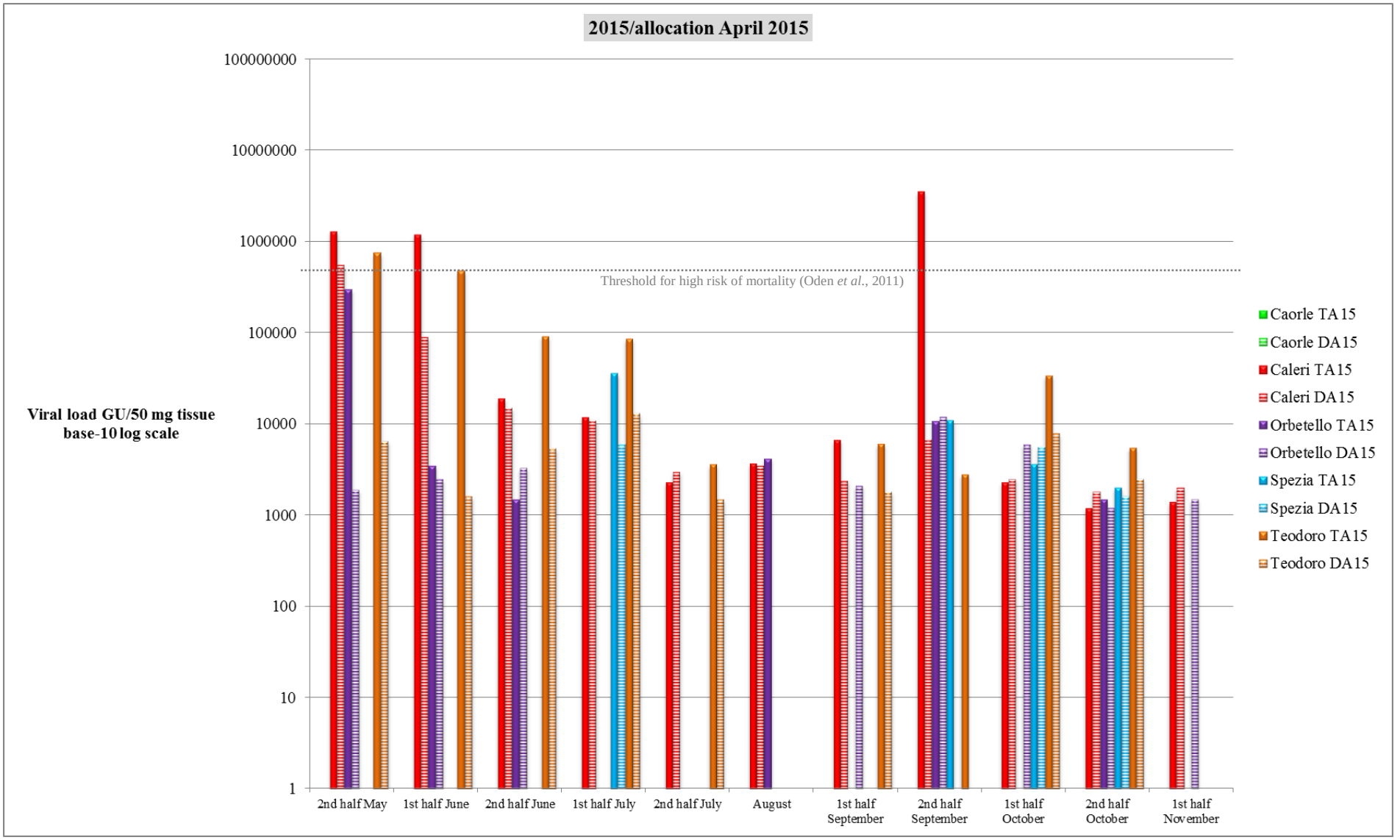


Fig.58C Mean viral load of OsHV-1 in positive samples in Italian sites in 2015 in spat allocated in April.

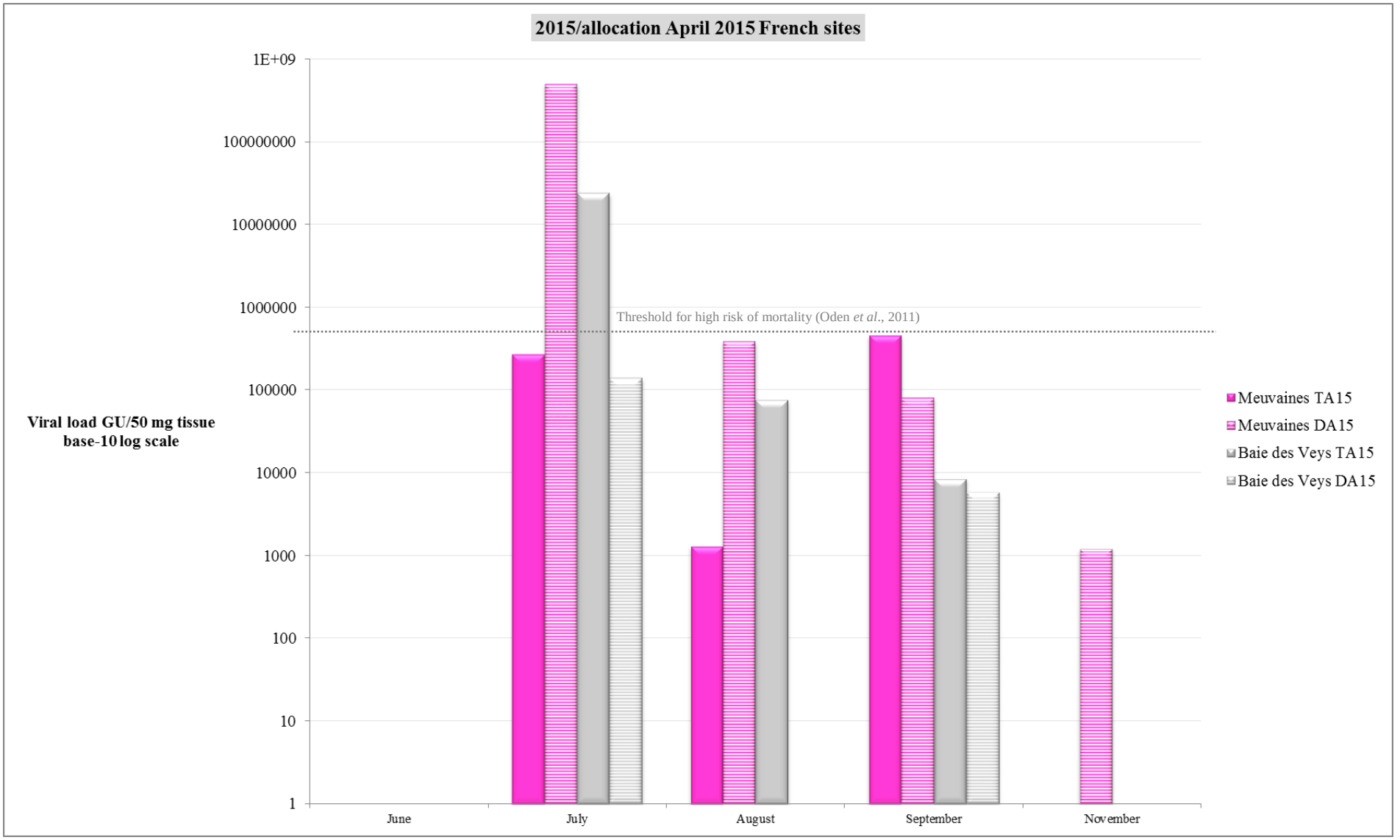


Fig.58D Mean viral load of OsHV-1 in positive samples in French sites in 2015 in spat allocated in April.

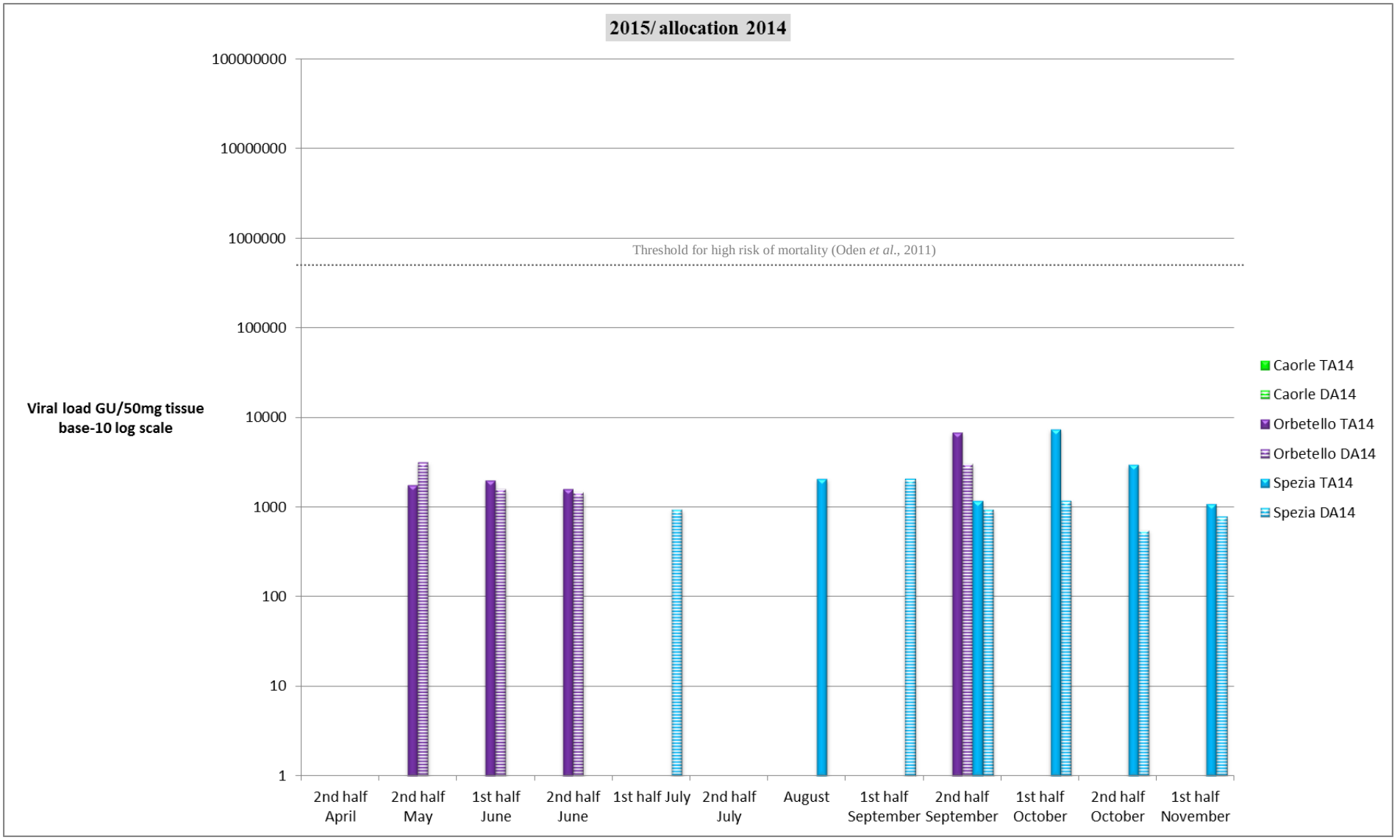


Fig.58E Mean viral load of OsHV-1 in positive samples in Italian sites in 2015 in individuals allocated in 2014.

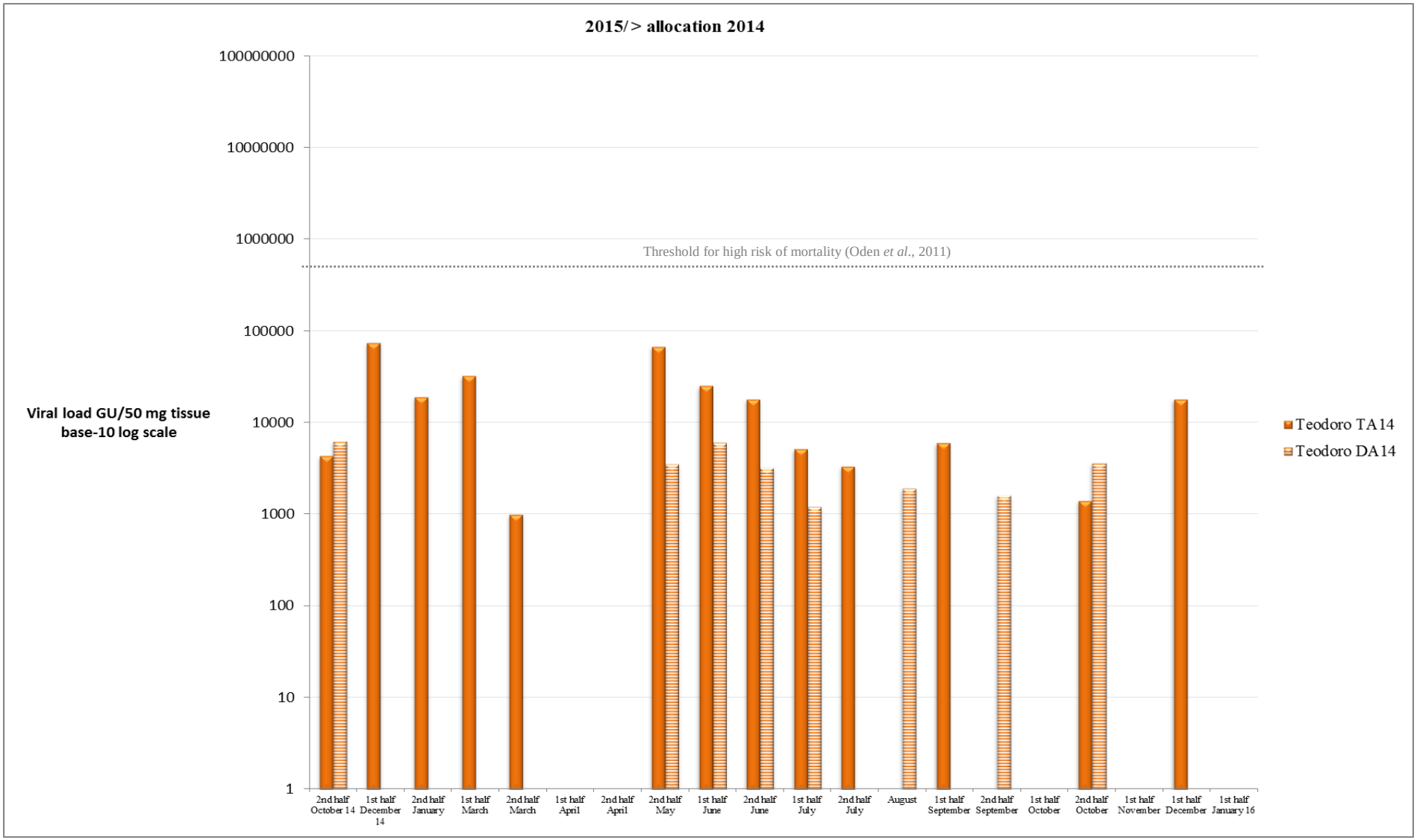


Fig.58F Mean viral load of OsHV-1 in positive samples in San Teodoro in 2015 in individuals allocated in 2014.

Prevalence and load of Vibrio aestuarianus in flesh

In the sites of Caorle, Giulianova, Gaeta, Orbetello, Olbia, Meuvaines, and Baie des Veys, *V. aestuarianus* DNA was never detected during all the survey. In the other four sites of Caleri, Varano, La Spezia, and San Teodoro, the bacterial DNA was only found at high loads in specific moment of the year, as evidenced in Figure 59. For the study of *V. aestuarianus* we grouped the results obtained from the individuals of the two allocations of 2014 (May and July), but we maintained them separated according to the ploidy: triploid 2014 (T14) and diploid 2014 (D14). In the months evidenced in the graph by an asterisk, data are available only for San Teodoro since in other sites no sampling was carried out. With the exception of San Teodoro, *V. aestuarianus* was not detected in spat in 2015, while it was present, in the first half of July and in the first half of October, in spat from Caleri, with loads reaching 3.2 x 10^8 GU/50 mg. In 2014, the bacteria was also found in spat individuals from Varano with a maximum load of 5.3 x 10^5 GU/50 mg. Finally, the presence of *V. aestuarianus* was evidenced in La Spezia, with moderate loads (4.5 x 10^4 GU/50 mg) in early summer and only in individuals allocated in 2014 > one year-old. The load was significantly higher in triploids ($p<0.05$).

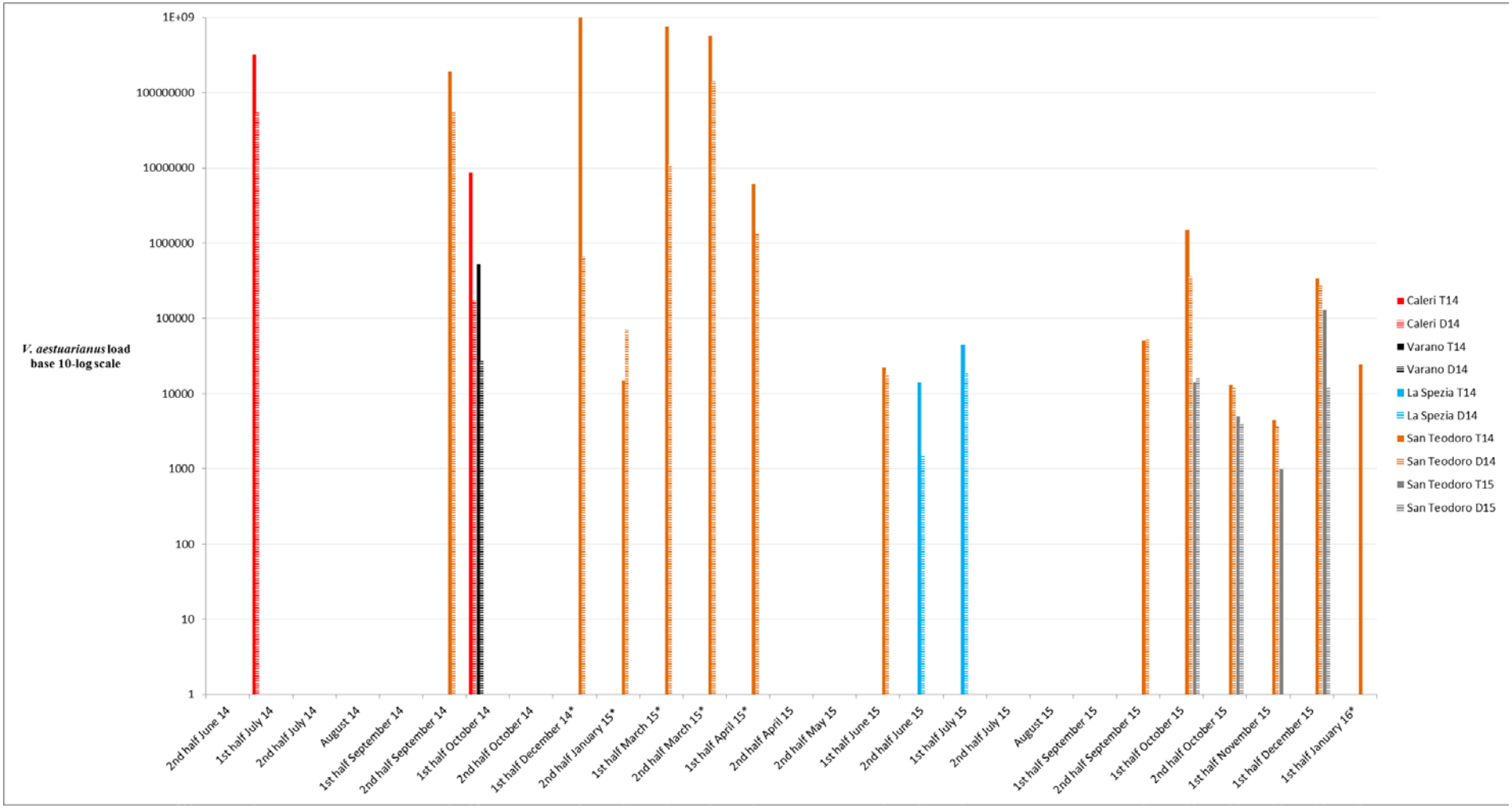


Fig.59 Mean load of *V. aestuarianus* in oyster flesh. *: results valid only for San Teodoro.

Bacteriological analysis

The total bacterial load, in moribund individuals analysed during 2014, was >104 CFU/mL of haemolymph in all the specimens. One hundred and fifty three strains were subjected to identification. In all individuals, collected from the various sites, the proportion of vibrios was comprised between 59 and 64% of the cultivable species, except for the site of Caorle, where vibrios represented only 35% of the isolated strains. Biochemical tests and MALDI-TOF gave inconclusive results for the non-vibrio strains. In the samples from Caleri, collected in July 2014, from San Teodoro, collected in September 2014, and from Varano, collected in October 2014, 30% of the *Vibrio* strains were identified as *V. aestuarianus* by the MALDI-TOF analysis, while our biochemical tests were not able to identify this species. The proportion of *V. splendidus*-related species between the isolated vibrios was around 80% in all samples except during July in Caleri and during September in San Teodoro, where the proportion decreased until 60% with the emergence of members of the *V. harveyi*-related species. However, the exact identification, at species level, of *V. splendidus* and *V. harveyi*-related species was not possible with these methods.

In 2015, the comparison between apparently healthy and moribund individuals evidenced significant differences ($p<0.05$) in the total bacterial load present in oyster haemolymph. A mean load of 2.5×10^2 CFU/mL of haemolymph was recorded in healthy specimens, while it reached 5.4×10^4 CFU/mL in moribund animals.

Histology

The presence of pathogenic parasites was excluded during histological examination that evidenced only aspecific lesions compatible with bacterial or viral diseases.

2.3.3. Discussion

The repeated insurgence of disease outbreaks in mollusc farming areas causes a serious economic impact, which claims the necessity to implement research activities to improve the comprehension of these phenomena. However, the exact reproduction of the natural conditions where these episodes occur is not possible in laboratory. Thus, during the present study, conducted for a period of two years, a survey was carried out in various different environments deploying oyster batches of the same origin and different ploidy, according to multiple-allocation campaigns.

Mollusc farming in lagoons is facilitated by the easy access for the operators, with a reduction of costs in comparison to the sites located far from the coastline. Furthermore, it usually guarantees a better protection of the equipment during storms than open waters sites. Moreover, as we observed during the present study, a faster growth is obtained in lagoons. The differences in growth observed between San Teodoro and other lagoons may be due to a better efficiency of the farming technique used *in loco*. These transition environments are characterised by eutrophic conditions able to provide high quantities of nutrients to filter feeder bivalves. For all these reasons, lagoons appeared to be the best kind of environment to farm oysters. Nevertheless, anomalous mortality events, defined as a mortality rate >20% between two samplings, associated with known oyster pathogens were mainly observed in this type of environment. In fact, sheltered and closed environments are usually correlated with higher OsHV-1 prevalence and mortality (Garcia *et al*., 2011). Moreover, farmers observed that the OsHV-1 risk seems to increase in fast-growing individuals as opposed to slow-growers and this affirmation was confirmed by various studies (Burge *et al*., 2007; Pernet *et al*., 2016).

In Caleri, in 2014, two peaks of mortality were observed: the first one during the first half of July and the second one in September (Figure 56A). Both were associated with viral loads exceeding the threshold defined by Oden *et al*. (2011), above which mortality is observed. These observations are consistent with the study conducted in the Thau Lagoon along the French Mediterranean coast in 2009, where mortality was observed between May and late September, with an interruption in July and August (Pernet *et al*., 2012). If we consider the graph of temperatures (Figure 53), we notice that the phenomenon observed in Caleri was not consistent with what observed in France during viral outbreaks, because in the former site mortality occurred with temperatures exceeding 25°C. In France, the peak of mortality associated with OsHV-1 was confirmed to occur just when seawater temperature

exceeded 16°C, as we stated in the batches placed in Normandy during the present study. In general a range of temperatures comprised between 16°C and 24°C has been defined for the occurrence of mortalities in Europe. Spat in France presented higher viral load than Italian one. However, in the Italian site of Caleri, where high viral loads were observed simultaneously with mortalities, the water temperatures exceeded 24°C in July. This observation is more consistent with the reports of OsHV-1 outbreaks that occurred between 23 and 25°C in Australia (Jenkins *et al.*, 2013; Paul-Pont *et al.*, 2013). Paul-Pont *et al.* 2013 showed that disease expression differed between sites, while temperature and salinity were comparable, suggesting that other environmental factors influence OsHV-1 infection. Nevertheless, in Caleri, mortality was highly correlated with prevalence and viral load, confirming a probable relationship between the virus and oyster mortality.

However, high loads of *V. aestuarianus* (Figure 59) were simultaneously present in the samples collected during mortality events in Caleri so that the responsibility of either pathogens may be questionable. In the same period, the net of the lanterns was highly obstructed and important quantity of mud was present in each compartment of the lantern inducing a low water renewal. These environmental conditions probably induced a stress in oysters that may have promoted the replication of both pathogens.

The environmental conditions in Orbetello, another Italian lagoon, during the same period were very similar to what observed in Caleri, with an important net obstruction and high water temperature. However, no anomalous mortalities were recorded and the loads of OsHV-1 and *V. aestuarianus* remained very low. In comparison to Caleri, where the density of wild individuals in the lagoon was extremely high, the density of wild population of Pacific oyster in Orbetello was very low. Moreover, even if oyster farming has been practiced in the past, this activity is stopped nowadays. As suggested in **Publication 1**, and on the contrary to what observed in Caleri, OsHV-1 is absent or present with a very low prevalence in the wild population living in this site. However, some individuals were infected by the virus.

In Caleri, in 2015, the seasonal trend of viral load was more consistent with literature, being maximal during the second half of May and the second half of September, when water temperatures where about 22°C. A mortality rate slightly higher if compared with other months was observed during May, the first half of June, and late September, corresponding to the months when the viral load exceeded the threshold defined by Oden *et al.* (2011), above which mortality occurs. Even if, in these cases, the increased mortality may be associated with OsHV-1 infection, the mortality rate was still moderate (≤20%). Since *V. aestuarianus* was not detected during 2015 in the site of Caleri, as opposed to 2014, when severe mortality occurs,

the differences in mortality rates between the two years may be explained by the influence of the presence of *V. aestuarianus*.

In the site of San Teodoro, OsHV-1 was found throughout the year, but viral load exceeded the threshold for mortalities only during May 2015 and was not associated with mortality. In this site, adult individuals were the most affected by disease and these events were always associated with high loads of *V. aestuarianus*.

Finally, the low viral loads of OsHV-1 (<8 x 10^3 GU/50mg) could not explain the mortalities observed in Varano in the first half of October 2014, but, on the contrary, the presence of *V. aestuarianus* was detected (5.3 x 10^5 GU/50 mg).

Mortalities were also observed in three sites located in open waters and gulfs during the two-year survey: Caorle, La Spezia, and Olbia. However, the association of these events with known infectious agents was not demonstrated and the hypothesis of the responsibility of abiotic factors was more probable. Both OsHV-1 and *V. aestuarianus* have not been detected in the two sites located in open water, Caorle and Giulianova, and in the Gulf of Gaeta. The episode that occurred in Caorle during 2014 was a single event, appeared in early autumn 2014 and it affected particularly the younger individuals, allocated in July, with a cumulative mortality >80%. In the same period, mussels present in this site suffered mass mortalities. Since an anomalous rain event, causing the extension of a freshwater layer from the rivers until the farming area as evidenced in Figure 54, preceded the phenomenon and since the presence of pathogens was not evidenced, and the bacterial load in the haemolymph of moribund individuals was comparable with what usually observed in healthy oysters (we observed an increase of the bacterial load in the haemolymph of moribund individuals during the other mortality events occurred during the present study), we can assume that the rain event, through the putative input of particulate in suspension and contaminants, and induction of low salinity, was responsible for the mortality event. A similar event, which was subsequent to abundant rain, was recorded in Olbia, in early October 2014, in absence of the detection of known pathogens.

Thus, it appeared that OsHV-1-induced mortalities were positively correlated with the neighbouring biomass of hosts and negatively correlated with seawater renewal. In Fact, these two parameters probably influence the concentration of infective particles, as observed by Petton *et al*. (2015).

The course of events in La Spezia is quite complex. In fact, during 2014, an increase in mortality (>20%) was observed during the first half of October in the batches allocated in July, but the virus was not detected in these batches. On the contrary, its presence was

ascertained in the batches allocated in May, with a load slightly exceeding the threshold for mortality. These observations may suggest that a source of stress (abiotic or biotic) contributed to the insurgence of mortality in younger individuals and to induce the replication of the virus in batches allocated in May. This hypothesis was confirmed during the chronic mass mortalities that affected the individuals allocated in La Spezia in 2015, when no known pathogen was detected. An environmental stressor or a source of pollution was therefore the best guess of this phenomenon, and the absence of mortality in older individuals may be explained by their greater resistance.

During the second year of the survey, by comparing individuals allocated in 2014 and individuals allocated in 2015, we confirmed that mortality was inversely related with age of oysters when facing the diseases. However, this affirmation was not completely true in San Teodoro, where adult individuals were mainly affected by mortality events. The course of these outbreaks differed between the young and the adult batches affected by disease, with acute events in young individuals and lower, but constant, mortality rates in adults, especially in autumn and winter.

The allocation date influenced also the mortality, with a greater survival in batches allocated in May and September in 2014, if compared with those allocated in July. The best survival was observed in 2015, with the allocation of spat in April. However, since no allocation was conducted in July 2015, it was not possible to conclude that this better survival, if compared with 2014, was due to the anticipation of spat allocation from May to April, or if it was the effect of other factors.

All the virus specimens found in infected oysters during the present survey were µVar, as defined by Segarra *et al*. (2010), except one specimen isolated in La Spezia that showed similarities with the Var genotype (Arzul, 2001). However, the viruses isolated in Caleri and even more in San Teodoro showed a moderate genetic diversity in comparison to the French µVar. This diversity was observed in Italy in wild populations (**Publication 1**), but not in France in farmed stocks. It is probable that, in conditions where virus replicates actively, as observed in Caleri and San Teodoro, and without inducing high mortalities (this assumption is true only for the year 2015 in Caleri), a coevolution is established between pathogen and host, leading to an increase of genetic diversity, as occurred in natural conditions. The presence of OsHV-1 was evidenced in various sites quite all over the survey, confirming that low viral loads are insufficient to cause the disease (Paul-Pont *et al*., 2014). It is not possible to affirm if the virus detected positive individuals was in a replicative phase, inducing a release of virions

in environment, or in a latent phase. However, as fluctuations in prevalence and viral loads were observed, with, in some cases, an apparent clearance of the virus in some batches, also observed by Whittingon *et al*. (2015), it is highly probable that viral reactivation in reservoir individuals is triggered by a stressful event, such as what observed in the site of San Teodoro, during disease associated with *V. aestuarianus* in autumn and winter. In any case, we showed that adult individuals represent a reservoir of OsHV-1.

The factor/s responsible of the higher mortality in 2014 if compared to 2015 was not clearly identified and cannot be attributable to OsHV-1, considering the fact that prevalence was slightly higher in 2015 and viral loads comparable. This statement confirms that the cause of mortality events in *C. gigas* spat and larvae is multifactorial, where OsHV-1 infection maybe necessary but not a sufficient cause, as asserted by Samain and McCombie (2008) and Petton *et al*. (2015).

Interestingly, out of mortality outbreaks *V. aestuarianus* was never detected, except in La Spezia between the second half of June and the first half of July, in batches not affected by mortality.

As shown in other studies (Pernet *et al*., 2012), the mortality observed in Italian sites in diploid individuals was generally higher than in triploid ones, but this tendency is inverted in adult individuals in San Teodoro. However, the high viral load observed in triploids, suggests that they may play a role in the diffusion of the disease, being able to survive to the infection, but being huge releasers of infective particles. In the specific events of mass mortality, occurred after anomalous rains in Caorle and Olbia, no significant differences between ploidies was observed, confirming that triploidy or genetic selection conducted by hatcheries may influence positively only the resistance to infectious diseases, in particular to OsHV-1.

2.3.4. Disease management

The present study was carried out to assess, in natural environment, the risk factors for oyster mortality insurgence in Italy, focussing on OsHV-1. In fact, to provide measures to overcome the problem of oyster mortalities, a priority is the identification of risk factors for disease. Management of mass mortality events associated with infectious agents is vital for aquaculture of *C. gigas*. Disease management relies on establishment and maintenance of disease freedom or control of established diseases. The guidelines are provided by the OIE, and by the European and national regulations. These measures comprise eradication, limitation of spread and prevention of disease introduction. However, most epidemiologic theory and management methods have been developed for terrestrial species and are unmanageable in marine environment, and especially for mollusc species. For instance, the control of environmental parameter is not feasible, vaccination is not practicable because of lack of adaptive immune system, and the openness and connectivity of marine systems induce high rates of epidemic spread. In the case of OsHV-1, viral particles are shed into water and disperse via water currents (Pernet *et al.*, 2012). Unfortunately, the minimum infective dose in natural conditions, an important parameter for the evaluation of risk of spread in environment, is unknown for OsHV-1. The distance over which the infection can be transmitted depends on hydrodynamics and decay rate. Virus decay in marine environments depends on environmental factors such as suspended particulates, salinity, temperature, bacterial flora, and grazing (Mojika and Brussaard, 2014). It has been shown that survival of OsHV-1 depends if it is free living or adsorbed to particulates (Paul-Pont *et al*., 2013; Evans *et al*., 2014). Whittington *et al*. (2015) demonstrated that the exposition of susceptible spat to seawater infected by OsHV-1 and then aged for 48 h does not induce mortality.

Thus, hatcheries and nurseries can be protected by prophylactic methods (Whittington *et al*. 2015; Helm *et al*., 2004). However, the use of aged water or chemotherapy (applicable essentially with bacterial diseases) is only suitable during the hatchery/nursery phase, requiring close systems. Moreover, genetic selection programs for the resistance of spat to specific pathogens seem to be a promising activity (Burge *et al*., 2007, Dégremont, 2011; Dégremont *et al*., 2015) breeding for resistance

Generally, a major risk of OsHV-1 introduction is more associated with spat collected from wild than with hatchery-produced individuals, even if certification of OsHV-1-free batches is commonly practised in France. In Italian farming areas, where wild population exists, the presence of OsHV-1 has been already evidenced (**Publication 1**). However, where

natural beds are not present, such as observed in most sites of the Tyrrhenian coast, and when new areas have been assigned to oyster farming activities, the use of certificated batches should be preferred in order to avoid the introduction of the viral pathogen.

Eradication of wild populations of oysters is an uncommon practice, because natural beds are very common. However, as adult farmed individuals, they represent dangerous reservoir of pathogens, including OsHV-1, by excreting the virus during stress or coinfection with other pathogens. Moreover, during these periods, animals are asymptomatic and may be transferred.

In general, the translocation of molluscs is the main way of spread and introduction of a pathogen in a new area. The difficulties in the control of the movements of live animals, with exchange of stocks between farming areas and countries, and the lack of traceability in oyster farming is a major limiting factor for the identification of epizootic sources routes of spread and application of control measures, even if these aspects are already regulated by the Directive 2006/88/CE.

Nevertheless, several management actions may be evaluated:

- culling of infected and disposal of dead individuals could contribute to control the disease spread. Under farming conditions, considering that moribund and dead infected oysters are likely to release OsHV-1 virions into the seawater, leading to horizontal transmission (Renault, 2011), attention may be paid on the treatment of waste. However, a daily collection of dead individuals and infected flesh is difficult to be applied, because the stock is not entirely controlled each day and it is difficult to visualise all the dead individuals.

- since oysters maintained in open waters usually do not suffer mortalities and OsHV-1 is only occasionally detected, as observed by Pernet *et al*. (2012), part of the production cycle, especially when oysters are more susceptible, may be conducted in open water, extending, in return, the duration of the farming cycle.

- a reduction of the stress induced by manipulations and handling may be determinant in mortality control during the periods of the year when oysters showed the maximum susceptibility. During the present study we evidenced that the greatest susceptibility occurs during late spring and early autumn.

- the period of spat allocation in the farming site seemed to be a relevant factor for the mitigation of mortalities. In fact, anticipation of the spat allocation in April/May or hindrance in September gave a better survival than deployment in July.

- since farming conditions provides persistent high density populations of host that offers ideal conditions for disease epidemics and because high densities are known to be a relevant

source of stress for farmed animals, the sustainability management of the ecosystem, with a limitation of stocks density, should be promoted.

- because of several aspects, mollusc culture shows higher similarities with plant cultivation than with zootechnics, and the establishment of fallow areas may be a suitable solution, especially in lagoons, to reduce the impact of diseases.

-if, nowadays, spat is usually certified OsHV-1-free at its arrival, this is not the case for *V. aestuarianus*, probably because this microorganism in not usually associated with spat mortality, but with disease in adults. However, as poor epidemiological data are available for this pathogen and since it was not found outside mortality events, attention should be paid on its risk of spread with the introduction of spat in a farming area.

2.4.Mortality events in farmed stocks

2.4.1. Description of a mortality event in farmed adult Pacific oysters C. gigas in Italy associated with the isolation of V. aestuarianus and Tenacibaculum sp.

Adult Pacific oysters, for their part, were known to suffer an increase of mortality during summer months in France (Costil *et al.*, 2005). The syndrome was termed “summer mortality”, resulting probably from the influence of different factors, mainly the physiological stress associated with gonadal maturation (Samain *et al.*, 2007), the presence of pathogenic vibrios such as *V. aestuarianus* and members of the Splendidus clade (Garnier *et al.*, 2007; 2008), and environmental conditions (Soletchnik *et al.*, 2007). However, since 2012, a recrudescence of mortalities affecting adult individuals of *C. gigas* has been observed in France (Repamo, 2013). The seasonality of the disease has changed with an extension and translation of the critical period later in the year (Repamo, 2013). If the viral disease in young individuals is characterised by an acute progress, on the contrary, during these phenomena a constant mortality rate is observed in adults from August to November, leading to a cumulative mortality of 65% in some farming areas and stocks. The aetiology of these events is uncertain and complicate, and different exogenous factors must be considered, together with intrinsic factors from the host.

Unfortunately, during 2013, the market-sized individuals reared in San Teodoro (Sardinia, Italy) have been in turn affected by mortality events. The local sanitary authorities estimated a loss of 50% of the production for the year 2013. The outbreak patterns were very similar to what observed in adult specimens in France: during autumn 2014, the first mortalities in adults appeared in September and lasted until January 2015, with a peak during December. These mortalities events generated a loss of about 40% of the marketable stock. With the aim to improve the comprehension of this phenomenon to better control the disease in future, a case study was carried out, with the collaboration of San Teodoro oyster farmers, and consisted in various diagnostic approaches.

Materials and methods

- *Sampling*

In December 2014, 15 symptomatic individuals of *C. gigas* (with a slowed reactivity of the adductor muscle) collected from a batch affected by anomalous mortalities in the San

Teodoro Lagoon and 15 asymptomatic ones, sampled in the same locality but from an apparently healthy batch, have been sent under refrigerated conditions to our laboratory where they were processed within 48 hours from sampling. All specimens were triploid, two years old and with a marketable size. The main environmental parameters met largely the tolerance ranges of the species, in particular the water temperature was 13°C at the sampling date, and no mortality was reported in other marine animal species in the lagoon area.

At their arrival at the laboratory, individuals were washed, opened and subjected to anatomo-pathological examination.

- *Histological examination*

A standard section of the body, containing the digestive gland, gut, gonads, and gills, was fixed for 48 hours in Davidson's fixative, together with excised parts of tissues showing gross lesions. Fixed tissues were then processed for routine histological examination and stained with haematoxylin and eosine (H&E). Giemsa and Gram's stains were also performed.

- *Bacteriological analysis*

Haemolymph of nine moribund individuals with gross lesions and the haemolymph of ten asymptomatic ones was drawn from the pericardial cavity using a 1 mL needle, after topic application of ethanol 70% at the insertion point to reduce the risk of contamination by external flora. Fourty µL of pure haemolymph and 40 µL of the 10^{-2} dilution of haemolymph in Zobell broth were spread on Zobell agar incubated for 48 h at 22 °C. A total count of the grown colonies was performed and the significance of the differences between asymptomatic and moribund individuals was evaluated through the Mann-Whitney test. For each oyster individual, the maximum number of colonies characterised by a different aspect in terms of colour and shape has been collected, and also when a low variability was present, at least twelve bacterial colonies were reisolated on Zobell agar to be characterised by molecular analysis. DNA extraction was performed by heating a colony placed in 250 µL of DNAse-free water for 10 min at 95°C. The first screening consisted in the discrimination of vibrios and then *V. splendidus*-related species among isolates. In fact, we focused on this group since some vibrio strains belonging to the Splendidus clade are known to be pathogenic for molluscs. Two consecutive Taqman® real-time PCRs targeting the 16S gene of *Vibrio* spp. (PCR1) and *V. splendidus* related strains (PCR2) (Nasfi *et al*., 2015) were performed. PCR1 uses the primer pair 16S1-F (5'GCGTAAAGCGCATGCAGGT3') and 16S1-R (5'AATTCTACCCCCCTCTACAG3'), and the probe 16S1-P

(5’TCAGATGTGAAAGCCCGGGG3’). Primers for PCR2 were SpF1 (5’ATCATGGCTCAGATTGAACG3’) and SpR1 (5’CAATGGTTATCCCCCACATC3’) and the probe SpProbe (5’CCCATTAACGCACCCGAAGGATTG3’). The not-Splendidus *Vibrio* strains were analysed by a third TaqMan® real-time PCR assay (PCR3) based on the amplification of part of the *dnaJ* gene (Saulnier *et al*., 2009). We used the primer pair *dnaJ*-F (5’ GTATGAAATTTTAACTGACCCACAA 3’) and *dnaJ*-R (5’ CAATTTCTTTCGAACAACCAC 3’) with the *dnaJ*-probe (5’ TGGTAGCGCAGACTTCGGCGAC 3’). Typical reaction volume of 25 µL contained 12.5 µL of Takara Premix Ex Taq™ 2X (Takara Bio Inc., Shiga, Japan), 0.5 µL of each primer (20 µM), 0.5 µL of probe (10 µM), 9 µL of DNA/nuclease-free water, and 2 µL of extracted DNA (replaced by 2 µL of DNA/nuclease-free water as negative control). The thermal cycling profile consisted of 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s (PCR1 and PCR3) or 62 °C for 30s (PCR2). The three assays were performed with Smart Cycler® (Cepheid, USA).

Colonies not identified as members of the Splendidus clade and *V. aestuarianus* were subjected to the sequencing of the *16S rRNA* gene, using the primer pair 16S-27F (5’AGAGTTTGATCMTGGCTCAG3’) and 16S-1492R (5’ACCTTGTTACGACTTCAC3’). For the conventional PCR, mixture was composed of 25 µL of Premix Ex Taq®2× Takara® (Lonza, Verviers, Belgium), 1 µL of forward primer (20 µM), 1 µL of reverse primer (20 µM), 21 µL of DNAse free water and 2 µL of DNA template. PCR was performed using a T100™ Thermal Cycler (Biorad, France). The thermal program was as follows: 10 s at 95 °C; 30 cycles of 10 s at 95 °C, 30 s at 55 °C, 40 s at 72 °C and a final extension of 3 min at 72 °C. PCR products were analysed with QIAxcel® Advanced System (Qiagen, Courtaboeuf, France) and those with the expected size were sent to Eurofins MWG Operon (Ebersberg, Germany) to be purified and subsequently sequenced on both strands. Forward and reverse individual gene sequences were aligned using ClustalW and sequencing errors were manually corrected. A consensus sequence was obtained and trimmed to an appropriate length. *16S rRNA* sequences were analysed with both BLAST® and NAST tool, based on NCBI and Greengenes databases respectively.

- *Quantification of OsHV-1 and* V. aestuarianus *in flesh*

For the quantification of *V. aestuarianus* and OsHV-1 in oyster tissues, two real-time PCR protocols based on TaqMan® technology (Applied Biosystems) were performed according to the method developed by Saulnier *et al*. (2009) and Martenot *et al*. (2010),

respectively. For the detection and quantification of *V. aestuarianus* in tissues the same primers and probe as for colony identification were used. The primer pair B3 (5'GTCGCATCTTTGGATTTAACAA3') and B4 (5'ACTGGGATCCGACTGACAAC3'), the B3-B4 probe (5'TGCCCCTGTCATCTTGAGGTATAGACAATC 3'), and an internal control (IC) probe (5' ATCGGGGGGGGGGGTTTTTTTTTTATCG 3') were employed for OsHV-1 detection and quantification. For each individual a pool of mashed tissues from adductor muscle, gills, heart, and mantle was subjected to DNA extraction using a QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands), following the manufacturer's protocol for blood or body fluids, except for elution performed in 60 µL Qiagen elution buffer AE. The PCR reaction volume and the thermocycle for both pathogens were identical to PCR1 and PCR3, with the addition, for the OsHV-1 assay, of 2 µL of IC solution (1.4×10^2 genome units (GU)/2µL) consisting of a synthesised sequence containing the complementary sequence of the forward and reverse primers at each end and internally the IC probe sequence. For the quantification of OsHV-1 and *V. aestuarianus*, standard curves were prepared according to the EURL for Mollusc Diseases Standard Operating procedures using dilutions of plasmidic DNA solutions for OsHV-1 and of bacterial DNA suspension for *V. aestuarianus*.

- *Experimental infections*

In order to assess the pathogenicity of the *Flavobacteriaceae* isolated from dying oysters during the present study, 45 adult triploid specimens of *C. gigas* apparently healthy were collected from a depuration basin in an oyster farm in Normandy (France) to be used in experimental infections simultaneously with 100 L of the seawater where they were submerged. At their arrival, oysters were maintained in emersion for 16 hours to boost successively the filtering capacities and the consequent efficiency of the anaesthesia. Then, the animals were anaesthetised by bathing 2 h in a solution containing 50 g/L of magnesium chloride dissolved in two-thirds of distilled water and one third of seawater (Suquet *et al*., 2009). The 45 individuals were randomly divided in six batches of five individuals and a batch of ten individuals. Infections were conducted on five specimens in triplicate and at two different concentrations of the inoculum: 10^4 (batches A, B, and C) and 10 CFU/µL (batches D, E, and F). Two hundred µL of bacteria suspended in sterile artificial seawater were injected into the adductor muscle of each of the five individuals of the six batches. A negative control, consisting in the ten remaining individuals (batch G), was included injecting animals with 200 µL of sterile artificial seawater (23 g/L NaCl; 1.5 g/L KCl; 1.23 g/L MgSO4,7H2O; CaCl2 0.3 g/L). Each infected batch was placed in a separate tank with 8 L of the seawater brought from

the depuration basin, while the negative controls were placed in a 16 L tank, and maintained at around 13 °C, under oxygenation conditions. Mortality was monitored every day for sixteen days. Dying animals were taken daily and 20 µL of haemolymph were drawn from each individual and spread in Zobell agar incubated for 48h at 22°C. The same protocol was applied on the survivals, at the end of the trial. A pool of minced tissues of each dead individual was used as described above to quantify OsHV-1 and *V. aestuarianus* by real-time PCR. At the end of the trial, significance of differences in mortality rate between the groups was evaluated using a Chi Square test. Results with a p-value <0.05 were considered statistically significant. The statistical tests were performed using R software, version 3.3.1.

Results

- *Anatomo-pathological and histological examinations*

Four of the symptomatic individuals were excluded from the analysis because they were dead at their arrival at the laboratory. Nine of the remaining specimens showed gross lesions, with multifocal green-yellowish areas on mantle and large areas of liquefactive necrosis in the adductor muscle as reported in Figure 60. These lesions were absent in asymptomatic specimens. The histological investigation confirmed the presence of multifocal necrotic areas, especially in the adductor muscle. A pronounced inflammatory response was observed with massive haemocytic infiltration around the lesions and an accumulation of haemocytes in the vascular sinuses. No parasitic infections were evidenced, but some lesions were associated with the invasion of tissues by filamentous bacteria, with colonies insinuated within muscle fibres Figure 61A. Gram's stain carried out on histological sections evidenced the presence of GRAM negative bacteria Figure 61B.

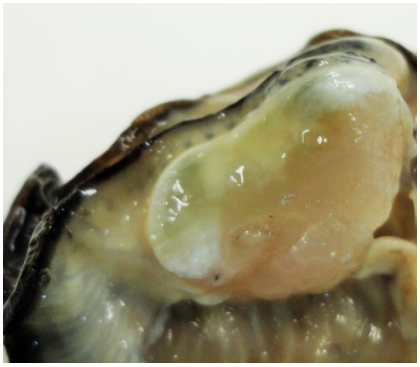


Fig. 60 *C. gigas*, liquefactive lesion in the adductor muscle of a moribund specimen.

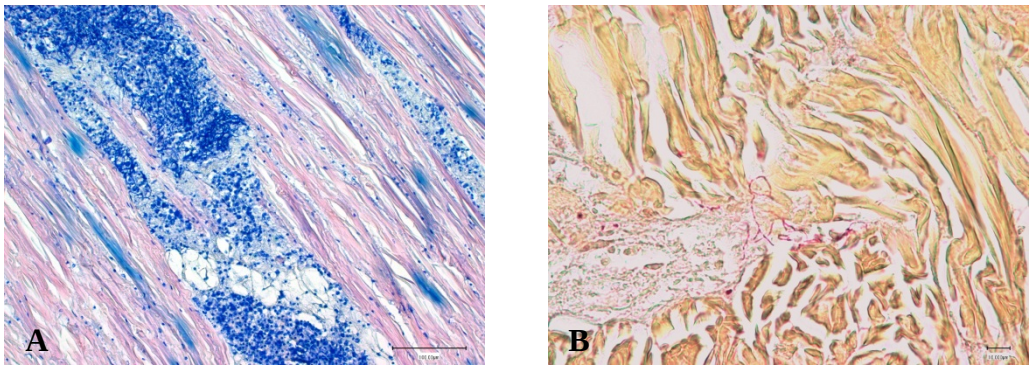


Fig 61 *C. gigas*, adductor muscle. A: filamentous bacteria in necrotic areas (Giemsa).; B: Gram- bacterial colonies (Gram).

- *Bacteriological analysis*

The bacteriological analysis conducted on oyster haemolymph evidenced a significant difference in the number of bacteria present in the haemolymph of moribund individuals (from 5.6 x 10^4 to 2.5 x 10^5 CFU/mL of haemolymph) when compared with asymptomatic ones (from 50 to 6 x 10^2 CFU/mL of haemolymph). *V. aestuarianus* was present in culture in 20% of the asymptomatic individuals and in 100% of the symptomatic ones. In asymptomatic individuals, 32% of the isolated strains were vibrios and different other genera were present, such as *Pseudoalteromonas* 18%, *Arcobacter* 18%, *Shewanella* 10%, and *Psychrobacter* 8%. Within *Vibrio* species, 78% of the strains were members of the Splendidus clade. In moribund individuals, the bacteria diversity in haemolymph was much lower with the isolation of only *Vibrio 62%*, *Arcobacter 18%*, and a member of the *Flavobacteriaceae* family. In both asymptomatic and moribund specimens, identification of some colonies was unsuccessful. These colonies were characterised by a yellow colour and an iridescent aspect as shown in Figure 62. Thus, the proportion of vibrios reached 62% in moribund individuals, but the proportion of *V. splendidus*-related species was comparable to that observed in healthy individuals. The *16s rRNA* sequence of the *Flavobacteriaceae* strains isolated in moribund oysters showed 99% of identities with *Tenacibaculum soleae* strains present in GenBank database and was deposited with the accession number KY765582.

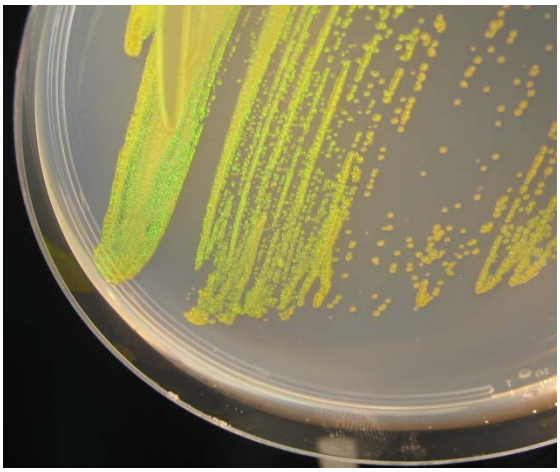


Fig 62 *Tenacibaculum* sp. isolated on Zobell agar from moribund oyster haemolymph.

- *Quantification of OsHV-1 and V. aestuarianus in flesh*

OsHV-1 was detected in 35% and 33% of asymptomatic and moribund individuals respectively. The highest viral load was observed in an asymptomatic individual, with 4.4 x 10^4 GU/50 mg of tissue, but below the limit of 4.4 x 10^5 GU/50mg in which mortalities usually occurs. On the contrary, the presence of *V. aestuarianus* DNA was evidenced in 100% of both moribund and healthy individuals. However, *V. aestuarianus* loads exceeded 10^8 GU/50mg of tissue in all the moribund individuals, while it remained below 6.5 x 10^5 GU/mg in the apparently healthy oysters.

- *Experimental infections*

Mortality occurred for the first time eleven days after infection, in the batch C, injected with the 10^4 CFU/mL bacterial suspension. At the end of the experimentation, 16 days after infection, the mortality rate with the inoculum 10^4 CFU/mL was 40% in batch A and B, and 60% in batch C, while only one individual died with the inoculum 10 CFU/mL in batch D. No mortality was observed in the control at the end of the experimentation. Thus, the cumulative mortality of 46.6% (Figure 63), observed at the end of the trial with the inoculum 10^4 CFU/mL, was significantly higher than the mortality observed with the inoculum 10 CFU/mL. No significant differences were observed between the inoculum 10 CFU/mL and the control.

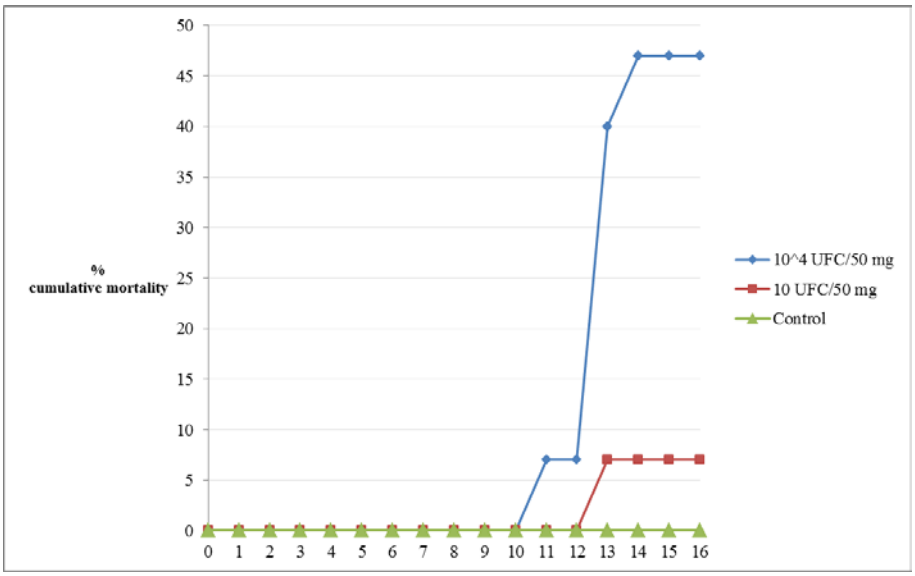


Fig. 63 Trend of cumulative mortality in the three groups after the experimental infection.

Gross examination of the eight dead individuals evidenced the presence of yellowish areas in the mantle (Figure 64) in all individuals. In three of these specimens, necrosis was observed in the adductor muscle (Figure 65). These lesions were very similar to what observed in oysters affected by mortalities in December 2014. Within the survivals, in one individual from the inoculum 10^4 CFU/mL and two individuals from the inoculum 10 CFU/mL the presence of yellowish areas in the mantle was highlighted at the end of the trial.

Fig. 64 *C. gigas*, presence of yellowish areas in the mantle after experimental infection with *Tenacibaculum* sp.

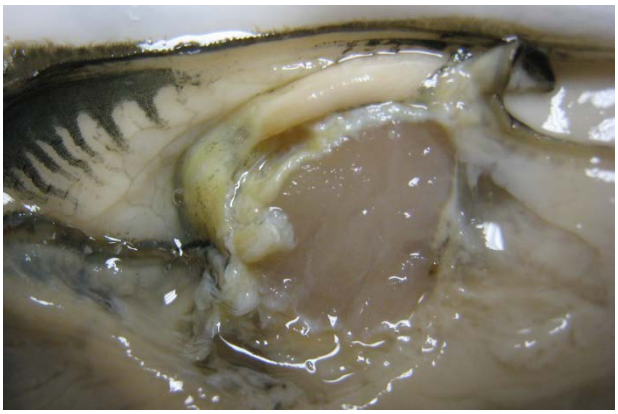


Fig. 65 *C. gigas*, presence of liquefactive necrosis in the adductor muscle after experimental infection with *Tenacibaculum* sp.

The presence of OsHV-1 at a low viral load (<10^2 GU/50 mg) was evidenced by real-time PCR only in one of the individuals dead after 13 days from infection with the inoculum 10^4 CFU/mL. *V. aestuarianus* was also detected, at an approximate load of 1.8 x 10^4 GU/50 mg, in only one specimen, which was the first that died eleven days after infection.

Finally, the bacteriological analysis conducted on dead oyster haemolymph showed the presence of the *Tenacibaculum* sp. in five of the seven individuals injected with the 10^4 CFU/mL inoculum and in the specimen injected with the 10^4 CFU/mL inoculum. The bacteria was also isolated in the three survivals with gross lesions at the end of the trial while it was not detected in the thaemolymph of the controls.

Discussion

The commercial sized stocks of Pacific oysters farmed in San Teodoro are periodically affected by mortality events during autumn and late winter, associated with characteristic yellowish lesions in mantle and adductor muscle.

During the present episode, parasitic and viral diseases were excluded. In fact, mortalities induced by OsHV-1 in adult oysters have been rarely reported (Batista *et al*., 2015), and the low viral load detected in a single oyster during the present study is unlikely to be associated with mortality (Oden *et al*., 2011).

Mollusc haemolymph is known to be non-sterile and characterised by a specific microbiota. Most of these bacteria are considered as commensal species and suspected to play a role in the host defence against pathogenic strains. In fact, they may produce and release in the haemolymph antimicrobial peptides able to inhibit the growth of pathogenic bacteria (Defer, 2010). However, in compromised hosts or under particular environmental conditions, strains from the commensal flora itself can act as opportunistic pathogens (Garnier *et al*.,

2007). During the present study we evidenced a lower diversity in the bacterial genera present in the haemolymph of moribund individuals, with a prevalence of *Vibrio* and *Arcobacter.* A similar observation was also made by Lokmer and Wegner (2015). Moreover, the bacterial load in haemolymph was significantly higher in moribund specimens. At present, the determinants of these microbial dynamics have not been identified yet, but they may be considered as a general declining health.

The pathogenicity of *V. aestuarianus* has been mentioned various times during this manuscript. In the site of San Teodoro, as detailed in the precedent chapter, the detection of high loads of *V. aestuarianus* was associated several times with mortality events. In the present case, in samples collected in December 2014, the loads of *V. aestuarianus* exceeded 10^8 GU/50mg of tissue in moribund individuals. Since the high pathogenicity of this bacterial agent has been demonstrated during experimental infections in precedent studies (Labreuche *et al*., 2006a; 2006b), its involvement in the presently described mortality event was the most probable hypothesis.

However, the systematic finding of a particular strain of *Flavobacteriaceae* in moribund individuals and the evidence of its presence in injured tissues raised the issue of its pathogenicity. According to the experimental trials conducted during the present study, a moderate pathogenicity was evidenced for this strain. Mortality appeared after a quite long period of more than ten days, when, with highly pathogenic strains, mortality occurs after 24-48 hours (Labreuche *et al*., 2006*b*; Travers *et al*., 2014). However, all the dead individuals showed the characteristic yellowish lesions and the inoculated bacteria was found in the haemolymph of most of them. The strain revealed high similarity with *Tenacibaculum soleae*. Tenacibaculosis is one of the more devastating infectious diseases of farmed marine finfish worldwide (Toranzo *et al*., 2005), causing ulcerative lesions and sometimes necrosis on the gills. In particular, *T. soleae* is a recently described species that causes high mortalities in commercially important species such as Senegalese sole (*Solea senegalensis*), Wedge sole (*Dicologoglossa cuneata*), Brill (*Scophthalmus rhombus*), and Turbot (*Scophthalmus maximus*) (Piñeiro-Vidal *et al*., 2008; López *et al*., 2010).

Interestingly, since December 2014, during all the mortality events occurred in the same periods of the year in San Teodoro, this *Tenacibaculum* sp. was isolated from haemolymph. However, its exact contribution in the development of the disease during these events is unknown. In fact, its appearance in oyster haemolymph may be only due to particularly favourable environmental conditions, when water temperature is around 13°C and in specific periods of the year that coincide with mortality events. In this case, it may play a role as

opportunistic pathogen, inducing necrotic lesions during infections with *V. aestuarianus*. Furthermore, during experimental infections, the stressful tank conditions may have also increased its pathogenicity. However, in natural conditions, when different potentially pathogenic strains coexist, cooperation between microorganisms is the most probable hypothesis of the development of the disease.

2.4.2. Description and investigation on a mortality event in spat during June 2016 in Normandy

In the last two years, the French monitoring program of mollusc mortalities, conducted by the REPAMO network, has recorded a decrease of the mortality events associated with OsHV-1 in spat in France (http://www.ifremer.fr/sante_mollusques/Documentation/). A common belief was that this recovery was due to the genetic improvement for disease resistance to OsHV-1. In fact, in the recent years, various breeding programs have been realised to enhance spat survival during summer mortality associated with the virus (see review by Degrémont *et al.*, 2015). However, in June 2016, a recrudescence of mass mortalities affected the young stocks of Pacific oysters in various French farming areas, exceeding 50% rate in numerous sites (Figure 66). Particular environmental conditions, able to promote the development of the disease or to inhibit it, may be involved in the kinetics of outbreaks. Nevertheless, another assumption may be the evolution of the virus, occurred to regain its advantage on the host thanks to genome mutations. In order to verify this last hypothesis, we analysed a batch of spat, collected in Normandy (France) in June 2016 during a mortality event, by sequencing 30 ORFs of OsHV-1. Histological and electron microscopy observations were also carried out to describe the tissue lesions and the virus morphology.

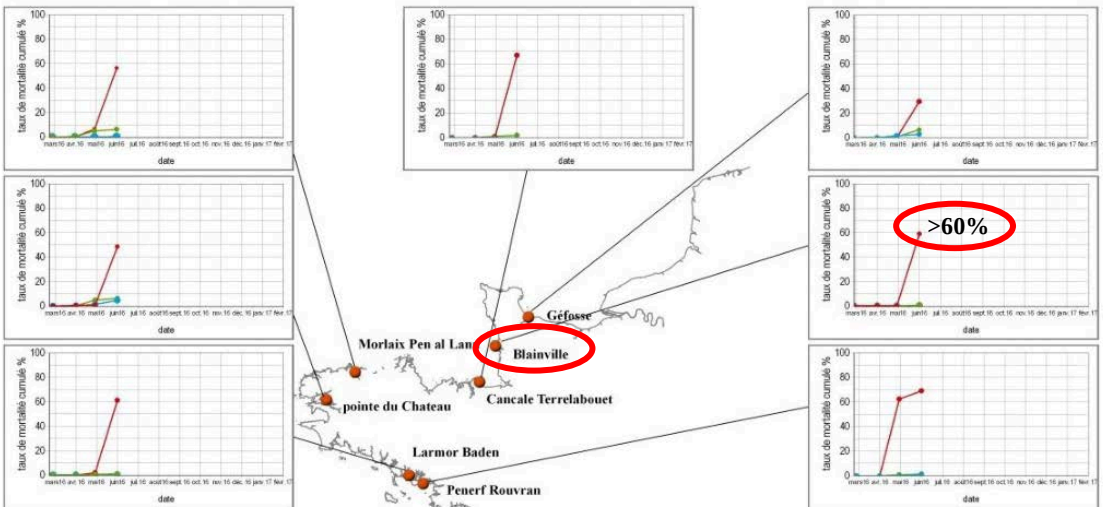


Fig. 66 Evolution of cumulative mortality rate in spat batches in June 2016 in the Northern French farming areas.

Materials and methods

- *Sample preparation for PCR assay and electron microscopy*

Diploid spat individuals, originating from natural collection in Vendée (France) in 2015, and with a mean length of 12 mm, were collected in Blainville/Mer (France) during the mortality event occurred in June 2016 . At their arrival, live individuals were shucked, four pools of five individuals were minced and 50±0.5 mg of flesh was subjected to DNA extraction according to Appendix B.1. The tissues of an additional pool of several individuals were disrupted with an Ultra-Turrax® (Staufen, Germany) homogeniser. Homogenate (1 ml packed volume) was immediately frozen at -80°C and sent to the laboratory of virology of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy) for electron microscopy observation.

- *Histology*

The occurrence of pathological conditions was evaluated by examining histological sections. Nineteen live individuals were shucked and immediately immersed in Davidson's solution for 24 hours. Fixed tissues were then processed by standard paraffin wax techniques, cut in 4±2 µm sections and stained with haematoxylin and eosin (H&E) for histopathological evaluations.

- *OsHV-1 quantification*

OsHV-1 quantification was performed on the four pools by real-time PCR as detailed in Appendix B.2. After referring to the standard curve, the viral loads were expressed in genome units GU/50 mg of oyster flesh.

- *Electron microscopy*

Tissue homogenate was diluted in distilled water 1:5 (v/v), vortexed and centrifuged at 4000 x g for 30 min at 8°C. The supernatant (3mL) was then clarified anew by centrifugating at 9300 x g for 30 min at 8°C. Eighty-five µL of supernatant were subsequently subjected to ultracentrifugation in Airfuge® centrifuge (Beckman Coulter s.r.l., Milano, Italy) with the A100 rotor, for 15 min at 20 psi (138 kPa) using microtubes with 150 µL volume and set up with specific adapters allowing the housing of 3 mm thick copper grid coated with formvar. The particules present in the sample pelleted directly on the grid and were then stained with a solution consisting in a 2% solution of sodium phosphotungstate (pH 6.8) for at least 3 min. Grids were dryed and observed with the G2 Spirit Biotwin (FEI Tecnai™, Thermo Fisher

Scientific) transmission electron microscope used at 85 kV at a magnification comprised between 19,000 and 34,500x. Microphotographs were taken with the Veleta (Olympus) camera.

- *ORFs sequencing*

The ORFs to be sequenced have been chosen referring to the genetic contents of the reference genome of OsHV-1 described by Davison *et al*. (2005), and on the basis of a previous study conducted by Martenot *et al.* (2013) that sequenced various ORFs of six OsHV-1 µVar strains isolated between 2009 and 2011, two in the same area in Western Normandy (Blainville). Four ORFs encode putative BIR proteins, six a RING domain, eight are supposed to be secreted, and twelve encode putative membrane proteins. The C region and a region overlapping ORFs42/43, discriminant for the definition of the µVar genotype (OIE, 2014) were also investigated. In addition, we used five primer pairs (see publication 2) to confirm the presence of the five large deletions observed in the µVar genotype in samples collected before 2011. The primer sequences are reported in Table 12. Amplifications of the different regions were performed on a single extract, according to the protocol described in Appendix B.3. The PCR products were subjected to QIAxcel® system analysis (Qiagen) to verify the amplification success and sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing both the sense and the antisense strands. The accuracy of the DNA sequencing was measured by the Phred quality score (Q score) and only scores above 30 were considered. The consensus sequence was determined by the alignment with ClustalW 1.81 (http://www.genome.jp/tools/clustalw/) and the comparison of both strand sequences. Our sequences were aligned with the sequences of Martenot *et al*. (2013).

Protein family - domain	Target in AY509253	Primer name	Sequence 5 '- 3'	Tm (°C)	Amplicon (bp)	Reference
BIR						
	ORF42	ORF42aFor	CAAGATGGAAGATGCACCAC	57.3	816	Martenot *et al*., 2013
		ORF42aRev	CCACCAATGTCTAAAGATCCC	57.9		
		ORF42bFor	GTCTACATTGAACTCTATCACC	56.5	572	
		ORF42bRev	CACTTCCGGATTTGAAGATATA	54.7		
	ORF87	ORF87For	GCATAACTCGTGGTATACCATTC	58.9	650	
		ORF87Rev	GTTCGTAATGTGTGTGGTTTCTTC	59.3		
	ORF99	ORF99For	GACAAAAATTCAAATCAGACAAGGG	58.1	903	
		ORF99Rev	CATTCAACCAAGTATTATCAACAAC	58.4		
	ORF106	ORF106aFor	TTGACTGTTCCGCTGTGAGT	57.3	883	
		ORF106aRev	GCAGGAGGATTGTGGTCATT	57.3		
		ORF106bFor	ATTGCCTCGTCATAGGCTGA	57.3	897	
		ORF106bRev	TGCAGAGCTTCATAACCCG	56.7		
Membrane proteins						
	ORF25	ORF25For	GTATGTGTGTACGCTTGGACG	59.8	824	
		ORF25Rev	TTCGGCATTCCCTTGAACAAATTC	59.3		
	ORF32	ORF32aFor	GATTTATAATAAACGTGGCAAGGGTG	60.1	901	
		ORF32aRev	TTTACCCATCGGTTTGGCATTGTT	59.3		
		ORF32bFor	GTTAAGAGATTGTGCCATGGGC	60.3	974	
		ORF32bRev	CTCCGGCAAATATGCAAGTGTAAT	59.3		
		ORF32cFor	TTCCGGTCAGATGACTATCAGT	58.4	721	
		ORF32cRev	AACATTGATGGGGATGTGACAG	58.4		
	ORF41	ORF41aFor	CCAATGGTTTTACCCCCTCAC	59.8	1144	
		ORF41aRev	TGGCTGTGGATAATTCCTTGAG	58.4		
		ORF41bFor	CCGAAGATAAGGCGGTGGTA	59.3	1168	
		ORF41bRev	CCAGATATGTATGCGGGTGTTT	58.4		
		ORF41cFor	CGCCGGATGATTACGCATGTA	59.8	1160	
		ORF41cRev	AGGTGCAAAAATGTCCCTTGTGT	58.9		
	ORF65	ORF65aFor	TGCCCTCCAATCAGGGTTTTC	59.4	1018	
		ORF65aRev	GCTCCACAAGCCAATCATGGT	59.8		
		ORF65bFor	TCGGCCAGTGTGGTACTGT	58.8	1386	
		ORF65bRev	TCACCTGGATGAACCGCCA	58.8		
	ORF68	ORF68aFor	GATTTACCACCCAGGCAGTTC	59.8	1270	
		ORF68aRev	CACCCAACAAGGTGGAGAAAC	59.8		
		ORF68bFor	GTTGCCATTAATCCACCAATGG	58.4	1232	
		ORF68bRev	TGGCGAGGGTACACAAGGA	58.8		
	ORF72	ORF72For	ACGTTTGAAGCCCGTGGAAAC	59.8	845	
		ORF72Rev	ACTTGTTGTCGCTTTGGTATCC	58.4		
	ORF77	ORF77aFor	ATATGCGGCCAAGGATTGACATT	58.9	1101	
		ORF77aRev	TTGTGTGACGGCCAAAACATTC	58.4		
		ORF77bFor	CCAATGACGATAACTCTAGAACC	58.9	1141	
		ORF77bRev	ACCAATGGAAAGGTATTAGGTTGG	59.4		
		ORF77cFor	GTGGACAAGTTGTAAACACCTGT	58.9	1108	
		ORF77cRev	CCCCGTCCTCAATTTAAGGC	59.3		
		ORF77dFor	CTTATTCGGAAGACCCTATACCA	58.9	1029	
		ORF77dRev	CTATCCGGTAGGTTTAATCCATTG	59.3		
	ORF80	ORF80For	CAAAAAGGCGCTCTTAAGCAG	57.9	471	
		ORF80Rev	AACGTAAATGAATGATCGACATGG	57.6		
	ORF84	ORF84For	CCTTCCCATACTTGGGGATTA	57.9	544	
		ORF84Rev	CTTTATATACTCTCCATTCGAAGG	57.6		
	ORF88	ORF88aFor	GTGATAACCCCAAAGAGGAAC	57.9	1020	
		ORF88aRev	CCCAGTCTATTATCCAGGTAC	57.9		
		ORF88bFor	ACCGTTCCTCAATCAGTCCC	59.3	711	
		ORF88bRev	GTTGGAATACCGCTCACCAC	59.3		
		ORF88cFor	GAAAGGTTCTGCGTCGACC	58.8	945	
		ORF88cRev	CTTTTTCAGCCAACCAAACATGG	58.9		
	ORF103	ORF103For	CATCAGCATCATTCCATCTACC	58.4	1476	
		ORF103Rev	GTAGATAACAAATCAAGCTAAGGC	57.6		
	ORF111	ORF111For	CATACTAAGATTGCCACAGCTC	58.4	1162	
		ORF111Rev	CATGAGAGTAGGCCATCGAC	59.3		

Table 12 Primer sequences used in PCR. The amplicon size corresponds to the expected size for the reference genome. Tm: melting temperature.

Protein family - domain	Target in AY509253	Primer name	Sequence 5 '- 3'	Tm (°C)	Amplicon (bp)	Reference
Secreted proteins	*ORF5*	ORF5aFor	AAGAGCGACTGGCCAGGAA	58.8	1166	Martenot et al., 2013
		ORF5aRev	CCACATCATCTAATTCGTCATACG	59.3		
		ORF5bFor	GGGAGATCTCGTTGTTATCGAAT	58.9	1110	
		ORF5bRev	TGGCCAGGAAACGATCGCAT	59.3		
	ORF13	ORF13For	TGGCCAGATGACGGTAGATG	59.3	649	
		ORF13Rev	CCGTATGTTAATTGTGCCCCAAA	58.9		
	ORF17	ORF17For	GCCGTGATGGTTACGCCAT	58.8	526	
		ORF17Rev	TTATTGCACCTTTCCCTTGTTCC	58.9		
	ORF39	ORF39For	TGTGGCTTCTGTGAGTTTTGAGT	58.9	745	
		ORF39Rev	GACCACGGGTGTGTAGGAA	58.8		
	ORF50	ORF50aFor	AAGATAGACCAGAGCTTGGAAG	58.4	1166	
		ORF50aRev	TGGGACTAGTGAGATATAAAGGG	58.9		
		ORF50bFor	TCGATCCGGCCAATCTTCCA	59.3	1073	
		ORF50bRev	ATGAATTTCCAAGATAAAGATATCGGGA	59.3		
	ORF74	ORF74For	TCAAGGAGCAGATTGAGATCTAC	58.9	595	
		ORF74Rev	TCTACCGGCGGACATTAGC	58.9		
	ORF83	ORF83aFor	CAGGCGCCAAGGAAACTCAT	59.3	776	
		ORF83aRev	GCCATTCTGCCAAGTTGTGG	59.3		
		ORF83bFor	TCTCGTTGGTGTATTGATTCACC	58.9	814	
		ORF83bRev	GATCAGCAAAAGTGTCATGGATG	58.9		
	ORF120	ORF120For	ATCATTGCGCATGTGTAAGGGA	58.4	600	
		ORF120Rev	ACAGTTTGGTGGAGGAGGTG	59.3		
RING fingers proteins	*ORF9*	ORF9aFor	TCCAGACATGTTTTCAGTTTGAGAT	58.1	1023	
		ORF9aRev	GGACCTGTTGATGTTGATATGAG	58.9		
		ORF9bFor	CCACCATTTAACACCTTTCTTGATA	58.1	1121	
		ORF9bRev	CAATGTAAAATTCTTCCCGGTCTG	59.3		
	ORF96	ORF96For	AAGAAATCCGCCAAGGGAAAGA	58.4	912	
		ORF96Rev	CATGTCTCTGCGCATTAGCG	59.3		
	ORF97	ORF97For	GGTTTCTCTTCCATACAGACCA	58.4	745	
		ORF97Rev	TGATGATACGAGCAACGCTTC	57.9		
	ORF117	ORF117For	GATGCACATCAGACACTGGC	59.3	1310	
		ORF117Rev	CACACACTTTTAAACCATAAAGATGAG	58.5		
	ORF118	ORF118For	GGTGAGATTAACCAATCAGCGAT	58.9	814	
		ORF118Rev	GATATCACCGGAACATGACGTTAT	59.3		
	ORF121	ORF121For	GGGAGTCTTACTGTACACATCTA	58.9	799	
		ORF121Rev	ACATCCAATGAAAACAGCCGGAA	58.9		
C region	*C region*	C2	CTCTTTACCATGAAGATACCCACC	58.9	709	Arzul (2001)
		C6	GTGCACGGATTACCATTTTT	56		
IAP	*ORF4s42/43*	IA1	CGCGGTTCATATCCAAAGTT	58.5	607	Segarra *et al*., 2010
		IA2	AATCCCCATGTTTCTTGCTG	58.4		
Deletions	*17,707 to 19,092*	Del1-F	AATTCAACGGGAAACAGACC	57.4	1313	Burioli *et al*., 2017
		Del1-R	TCTCCATTTCCTTGGACTGC	58.6		
	52,253 to 52,858	Del2-F	ATACGATGCGTCGGTAGAGC	58.7	458	
		Del2-R	AGAGCGATGGCAAAATTACG	58.7		
	67,973 to 68,572	Del3-F	ACATTTCATCATGCCCAAGG	58.3	926	
		Del3-R	TTCCGGGATAAATAGCATGG	58.7		
	93,120 to 96,669	Del4-F	ACATGTTCATCTGCCACAGG	58.6	1250	
		Del4-R	AAACCACCTGCCATACTTGG	58.6		
	175,018 to 175,743	Del5-F	TCTTGGGAATGGTGAAGAGC	58.6	772	
		Del5-R	TTTCCAATTCCGTCTTCTCG	57.9		

Table 12bis Primer sequences used in PCR. The amplicon size corresponds to the expected size for the reference genome. Tm: melting temperature.

Results

- *Histology*

Tissue lesions were observed in sixteen individuals with different degrees of severity. At low magnification (Figure 67), a severe haemocytosis was evidenced in the connective tissue of mantle and gills, with a very marked degree of invasion.

Adductor muscle showed multifocal necrotic areas with degeneration of myocytes and haemocytes (Figure 68A). In addition, an anomalous infiltration of blast-like cells and normal haemocytes between the muscular fibres was also observed (Figure 68B).

Several oysters presented a severe atrophy of the diverticular epithelium leading to the lumen enlargement. Haemocytosis was detected in tubules with the liberation of hyalinocytes in the lumen.

The occurrence of a conspicuous number of abnormal haemocytes, mainly blast-like cells, throughout the connective tissue characterised the pathological condition (Figure 69).

The typical architecture of the connective tissue of the mantle was lost and replaced by the proliferative cells (Figure 69) and bundles of fibres, as previously described by da Silva *et al*. (2008) in *Ostrea edulis* infected by OsHV-1, were present. Most of the proliferative cells showed a marginated chromatin, pycnotic nuclei or nuclear fragmentation (Figure 70). Degenerated large eosinophilic cells, characterised by an irregular shape and an indistinct nucleus, were also present throughout the connective tissue.

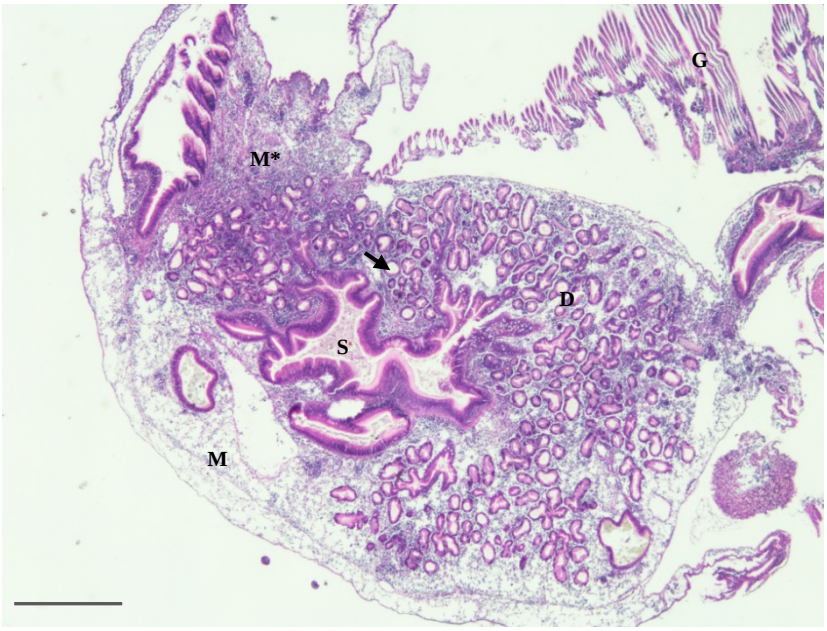


Fig. 67 *C. gigas*, spat specimen. D: digestive diverticula; G: gills; M: mantle; M*: haemocytic infiltration in mantle; S: stomach; arrow: enlarged lumen. (H&E). Scale bar = 750 µm.

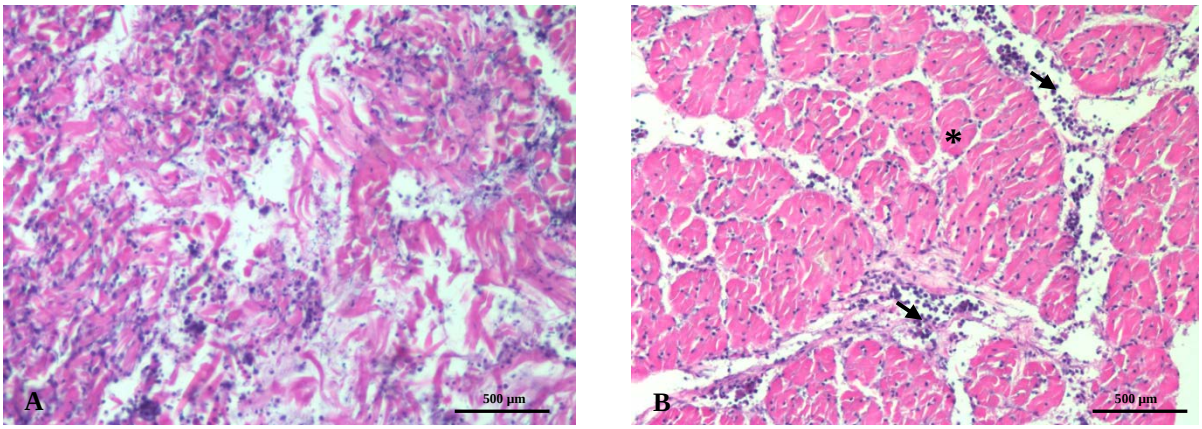


Fig. 68. *C. gigas*, adductor muscle. A: necrotic area with the degeneration of myocytes and haemocytes; B: haemocytes infiltration (arrow) within muscular fibres (asterix). (H&E). Scale bar = 500 µm

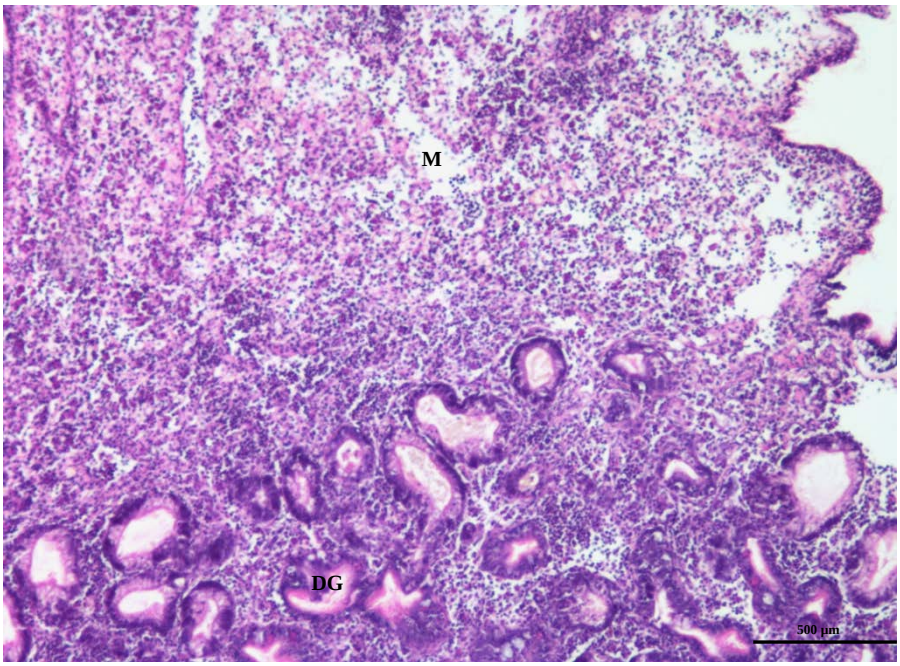


Fig. 69. *C. gigas*, mantle and digestive gland. Severe infiltration of the connective tissue by proliferated cells. DG: digestive gland; M: mantle. (H&E). Scale bar = 500 µm

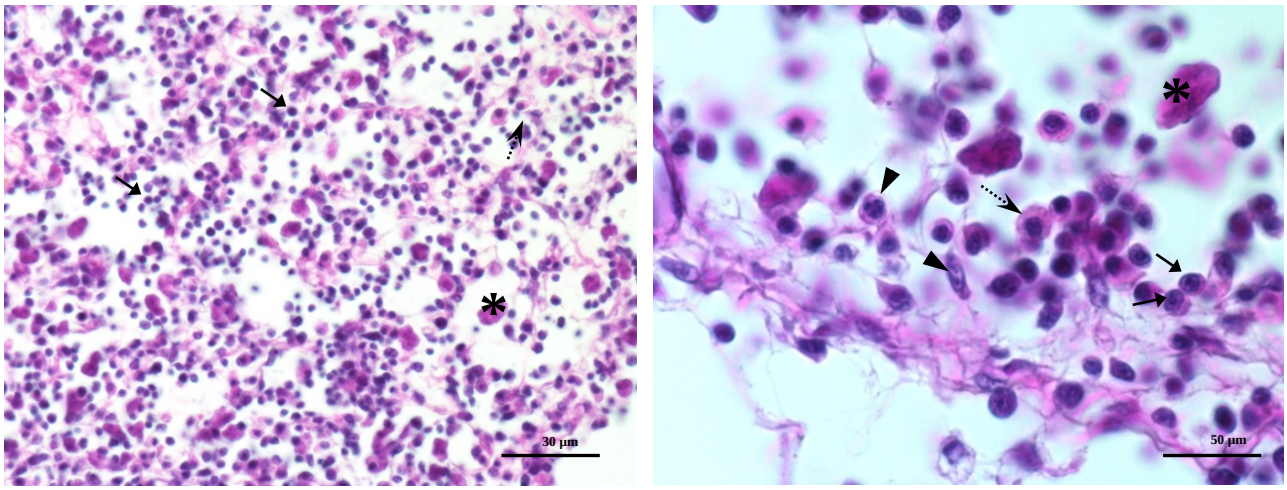


Fig. 70 *C. gigas*, mantle. A: severe infiltration of the connective tissue by proliferated blast-like cells (arrow) and large eosinophilic degenerated cells (asterix), normal haemocyte (dotted arrow) (H&E). Scale bar = 30 µm ; B: Degenerated eosinophilic cell (asterix); karyorrhexis (arrowhead), marginated chromatin (arrow) in proliferated blast-like cells and normal haemocyte (dotted arrow). (H&E). Scale bar = 50 µm

- *Electron microscopy*

No enveloped viral particle was observed with electron microscopy, but the presence of icosahedral capsids, with a conformation congruent with herpesviruses, was confirmed (Figure 71). The measurements of 25 capsids revealed a mean diameter of 104.81 nm.

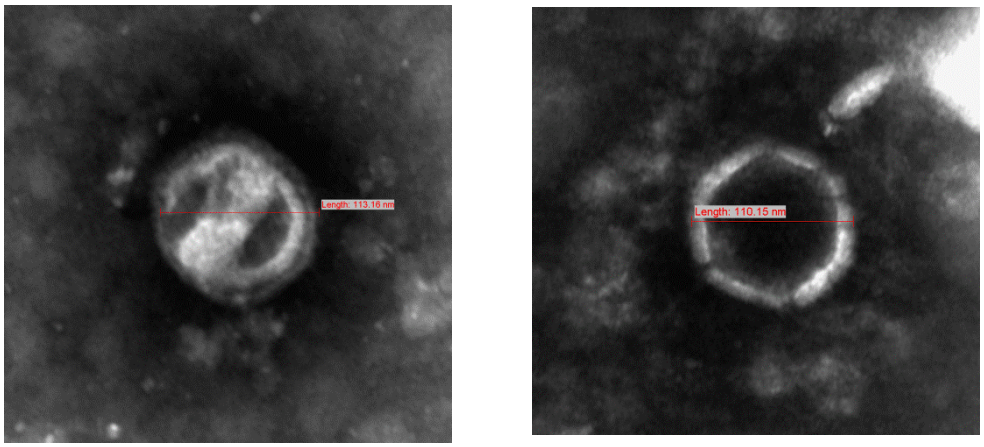


Fig. 71 OsHV-1 capsids observed in TEM after negative staining (A. Lavazza).

- *OsHV-1 quantification and ORFs sequencing*

The four pools of oysters spat tested positive for OsHV-1. The viral loads were comprised between 1.4 10^8 and 1.4 10^{10} GU/50 mg.

The sequencing of the C2/C6 region and ORFs42/43 confirmed that the isolated virus was a µVar variant. The presence of the five deletions, already observed in the µVar genotype, was confirmed in the present study. No variation of the sequence of ORFs encoding BIR proteins was evidenced in our sample. Within proteins characterised by the presence of RING finger domain, only ORF118 presented the substitution of one nucleotide, without codon change. In ORF5, supposed to be a disrupted gene encoding a presumably non-functional secreted protein according to Davison *et al*. (2005), four substitutions of nucleotides were evidenced. As observed in ORF 5, another putative disrupted gene, ORF 65, showed a significant variability with the substitution of three nucleotides. The main variability was observed among membrane proteins: one codon substitution was present in ORFs 25, 32, 80, 88, and 111, and two in ORFs 41 and 103. However, the most important variation consisted in the 11-codon shortening of the polypeptidic chain encoded by ORF 72 and by the substitution of the last 22, as a consequence of the insertion of two nucleotides. The alignments of the sequences obtained during the present study with those of Martenot *et al*. (2013) are reported in Appendix D.8.

Discussion and conclusion

The acute course of viral disease in young oyster specimens and the rapid degradation of tissues often preclude the possibility to conduct histological observations from outbreaks occurred in field. In fact, most of the observations performed in previous studies derived from experimental infections. During the present study, live individuals with high viral loads were sampled and studied. Haemocytosis, concerning mainly blast-like cells, and the degeneration of myocytes and large eosinophilic cells in the connective tissue of mantle appeared the most characteristic pathological lesions. A continuous proliferation of immune cells is also observed in Epstein-Barr virus (EBV) infection (Tsurumi *et al*., 2004). EBV is a gamma herpesvirus and the causative agent of infectious mononucleosis in humans. During the lytic replication, the infected B lymphocytes proliferate resulting in lymphoblastoid cell lines (Fields *et al*., 2002). At the moment, during OsHV-1 infection in oysters, it is not clear if the cell proliferation is a host defence reaction against the infectious agent or if the virus directly orchestrates this cell multiplication. In any case, this severe haemocytosis causes a condition that can easily result in a rapid death of affected animals.

The electron microscopy observations did not evidence enveloped particles, however the treatment used for negative staining may have damaged the lipid membrane, a condition often observed with herpesviruses. Nevertheless, the capsid architecture confirmed that it belonged to the order *Herpesvirales*. The capsid diameter (104.81 nm) of this OsHV-1 was slightly smaller than the estimation of Davison *et al*. (2005) for OsHV-1 reference (116 nm). Several studies reported a smaller OsHV-1 capsid diameter, comprised between 70 and 85 nm (Le Deuff and Renault, 1999; Renault *et al*., 2001). These differences observed among the various studies are probably due to the use of different protocols. In fact, we performed a negative staining, while Davison *et al*. (2005) used cryo-electron microscopy, and in all other studies the observations were carried out on tissues after a fixation step that is known to provoke the contraction of the structures.

Genetic investigations confirmed that the virus infecting spat in Normandy (France) in June 2016 was a µVar genotype. No variation was observed in the C region when compared to the µVar described by Segarra *et al*. (2010). If compared to the variants isolated by Martenot *et al*. (2013) between 2009 and 2011, a total of eleven ORFs presented single nucleotide mutations. Several nucleotide substitutions occurred in the two disrupted genes (ORFs 5 and 65), but since they are supposed to be non-functional we did not evaluate the effect of these mutations on the codon chain. Among the putative functional investigated ORFs, as expected, those encoding membrane proteins showed the highest number of variations with codon

substitutions in seven of them and huge changes in the eighth (ORF 72). Given that the transmembrane motif was maintained, it is hard to affirm if the original properties of the encoded protein were maintained or not. The transmembrane proteins, present on the viral envelope, play a critical role in the virus entry into the host cell. Thus, they are subjected to a high selection pressure, inducing an elevated genetic diversity that plays an important role in the evolution of virus virulence (Vigerust and Shepherd, 2007). In fact, by comparing ORFs encoding membrane proteins of OsHV-1 isolated in oyster spat from the same area in Blainville, but at a temporal distance of five to eight years, we detected an important evolution of the virus genotype. The rearing conditions, with a higher density of sensitive oyster specimens, may promote a high replication rate of the virus, probably inducing a drastic increase in its evolution rate.

However, at the moment, further studies are needed to evaluate if these mutations may play a role in the increase of virus virulence, in particular to understand how they could influence the relationship between the host cells and the virus during both the attachment and penetration phases.

GENERAL CONCLUSIONS & PERSPECTIVES

In a period of expansion of oysters farming in Italy, the present work aimed to contribute to this challenge approaching several important aspects connected with oyster health management.

Firstly, we evidenced the presence of natural populations of *C. gigas* along the Italian coasts and showed differences in the distribution and density population between Adriatic and Tyrrhenian Sea. The presence of OsHV-1 infecting these wild populations was detected in all the Adriatic beds, demonstrating high diversity of genotypes, and showing that these individuals may play a role as reservoir of infection in farmed stocks allocated in the same sites. No other potential reservoir hosts have been evidenced during the investigation conducted in other mollusc species. However, we need to extend this study during a larger part of the year to minimise a possible effect of seasonality.

The obtainment of the complete sequence of OsHV-1 µVar genome represents a significant goal reached during the present work. In particular, this result will permit the exploration of virulence factor in future, a better use of transcriptomics, and the development of new specific diagnostic tools.

Nevertheless, some important aspects related to OsHV-1and relevant for the control of the disease need further investigation, such as to determine if vertical transmission of OsHV-1 and latency are a reality or not.

The complete comprehension of the mechanisms at the origin of the mortality events, observed during the two-year survey, is arduous but the study allowed to observe and obtain highly useful information on oyster mortalities and associated pathogens, specifically in the Italian context. In particular, the impact of *V. aestuarianus* seems to be relevant in some areas and knowledge enhancement is necessary. A potential new pathogen for oysters, a *Tenacibaculum* strain, has been decribed. Thanks to this information, several possible measures for the disease management were evidenced.

However, some aspects need to be clarified. For instance, the interactive effects of pollution, climatic changes and infectious diseases need to be deepened. Moreover, the involvement of pathogenic bacteria in mollusc mortalities has been evidenced in a conspicuous number of studies (Paillard *et al*., 2004; Garnier *at al*., 2007) but these topics must be considered under new perspectives. In fact, we observed an influence of seasonality and health status on species diversity and microbial population structure. Over the last couple

of decades, it has become clear that microbiota are of vital importance for homeostasis of animals (McFall-Ngai *et al*., 2013). Most of these bacteria are considered as commensal species and suspected to play a role in the host defence against pathogenic strains. However, since most of these species are uncultivable and a high number of strains needs to be identified contemporary to guaranty a statistical significance over the year, a metagenomics approach is necessary.

A further topic of development should be the obtainment of mollusc cell lines, to date unavailable, but necessary for the study of viruses and toxicological evaluations.

In order to deepen the present knowledge, and with the aim to implement new tools to optimise the diagnotics of the most important diseases of Pacific oysters, we are applying for several and new research fundings to continue and develop our investigation.

BIBLIOGRAPHY

Bibliography

Adams K.L., Wendel J.F., 2005. Novel patterns of gene expression in polyploid plants. Trends Genet., 21: 539-543. doi: 10.1016/j.tig.2005.07.009.

Afonso C.L., Tulman E.R., Lu Z., Oma E., Kutish G.F., Rock D.L., 1999. The genome of *Malanoplus sanguinipes* entomopoxvirus. J. Virol., 73: 533-552. PMID: PMC103860.

Akula S.M., Pramod N.P., Wang F.Z., Chandran B., 2002. Integrin α3β1 (CD49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) entry into the target cells. Cell, 108:407–419. doi: 10.1016/S0092-8674(02)00628-1.

Alderman D.J., Gras P., 1969. "Gill disease" of Portuguese oyster. Nature, 224: 616-617.

Allen S.K., Stanley J.G., 1978. Reproductive sterility in polyploid brook trout, *Salvinelus fontinalis*. Trans. Am. Fish Soc., 107: 474-478.

Allen S.K.Jr., Downing S., 1986. Performance of triploid Pacific Oysters, *Crassostrea gigas* (Thunberg). I. Survival, growth, glycogen content, and sexual maturation in yearlings. J. Exp. Mar. Biol. Ecol., 102: 197-208. doi: 10.1016/0022-0981(86)90176-0.

Arzul I., 2001. Herpès virus infectant les bivalves marins: détection, génome et transmission. Thèse.

Arzul I., Garcia C., Joly J.P., Lupo C., Travers M.A., Francois C., Chollet B., 2013. Report of the Annual Meeting and 9th Combined Technical Workshop of the National Reference Laboratories for Mollusc Diseases. Rochefort and La Tremblade, 18–21 March 2013.

Arzul I., Nicolas J.L., Davison A.J., Renault T., 2001*a*. French scallops: a new host for Ostreid Herpesvirus-1. Virology, 290 (2), 342–349. doi: 10.1006/viro.2001.1186.

Arzul I., Renault T., Lipart C., 2001*c*. Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. Dis. Aquat. Org., 46: 1-6. doi : 10.3354/dao04001.

Arzul I., Renault T., Lipart C., Davison A.J., 2001*b*. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. J. Gen. Virol., 82: 865-870. doi: 10.1099/0022-1317-82-4-865.

Arzul I., Renault T., Thébault A., Gérard A., 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. Virus Res., 84(1-2) : 151-160. doi: 10.1016/S0168-1702(02)00007-2.

Auffret M., 1989. Comparative study of the haemocytes of two oyster species: the European flat oyster, *Ostrea edulis* (Linnaeus, 1750) and the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793). J. Shellfish Res., 8(2): 367-373.

Babior B.M., 1984. Oxidants from phagocytes: agents of defense and destruction. Blood, 64: 959-966. PMID: 6386073.

Bachère E., Diego Rosa R., Schmitt P., Poirier A.C., Merou N., Charrière G.M. Destoumieux-Garzón D., 2015. The new insights into the oyster antimicrobial defense: cellular, molecular and genetic view. Fish Shellfish Immunol., 46: 50-64. doi: 10.1016/j.fsi.2015.02.040.

Bachère E., Gueguen Y., Gonzalez M., de Lorgeril J., Garnier J., Romestand B., 2004. Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and

the oyster *Crassostrea gigas.* Immunol. Rev., 198: 149-168. doi: 10.1111/j.0105-2896.2004.00115.x.

Barber B.J., 2004. Neoplastic diseases of commercially important marine bivalves. Aquat. Living Resour., 17: 449-466. doi: 10.1051/alr:2004052.

Batista F.M., Arzul I., Pépin J.F., Ruano F:, Friedman C.S., Boudry P., Renault T., 2007. Detection of Ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review. J. Virol: Methods, 139: 1-11. doi: 10.1016/jviromet.2006.09.005.

Batista F.M., Leitão A., Huvet A., Lapègue S., Heurtebise S., Boudry P., 2005. The taxonomic status and origin of the Portuguese oyster *Crassostrea angulata* (Lamark, 1819). The 1st International Oyster Symposium, 13-14 July 2005, Tokyo, Japan.

Batista F.M., López-Sanmartín M., Grade A., Morgado I., Valente M., Navas J.I., Power D.M., Ruano F., 2015. Sequence variation in Ostreid herpesvirus 1 microvar isolates detected in dying and asymptomatic *Crassostrea angulata* adults in the Iberian Peninsula: Insights into viral origin and spread. Aquaculture, 435: 43-51. doi: 10.1016/j.aquaculture.2014.09.016.

Baud J., Bédier E., Beliaeff B., Boudry P., Pernet F., Renault T., Robert R., 2012. Les surmortalités des naissains d'huîtres creuses *Crassostrea gigas* - Journée d'information et d'échanges du 18 janvier 2012. Note Synthèse.

Beattie J.H., Davis J.P., Downing S.L., Chew K.K., 1988. Summer mortality of Pacific oysters. Am. Fish. Soc. Spec. Publ., 18: 265–268.

Beaumont A.R., Fairbrother J., 1991. Ploidy manipulation in molluscan shellfish: a review. J. Shellfish Res, 10: 1-18.

Ben-Porat T., Tokazewski S.A., 1977. Replication of herpesvirus DNA II. Sedimentation characteristics of newly synthesized DNA. Virology 79, 292–301. doi:10.1016/0042-6822(77)90356-7.

Bentz J., 1993. Viral Fusion Mechanisms. Boca Raton, FL: CRC Press. 529 pp.

Beutler B., 2004. Innate immunity: an overview. Mol. Immunol., 40: 845–859. doi: 10.1016/j.molimm.2003.10.005.

Birchler J.A., Yao H., Chudalayandi S., 2007. Biological consequences of dosage dependent gene regulatory systems. Biochim. Biophys. Acta., 1769/5-6): 422-428. doi: 10.1016/j.bbaexp.2006.12.002.

Bitton G., 1975. Adsorption of viruses onto surfaces in soil and water. Water Res., 9: 473-484. doi: 10.1016/0043-1354(75)90071-8.

Boening D.W., 1999. An evaluation of bivalves as biomonitors of heavy metals pollution in marine waters. Environ. Monit. Assess., 55: 459-470. doi: 10.1023/a:1005995217901.

Bouchet P., Gofas, S., 2012. *Crassostrea* (Sacco, 1897). In: MolluscaBase (2016). Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=138297 on 2017-01-03.

Boudry P., Heurtebise S., Collet B., Cornette F., Gérard A., 1998. Differentiation between populations of the Portuguese oyster *Crassostrea angulata* (Lamark) and Pacific oyster *Crassostrea gigas* (Thunberg), revealed by mtDNA RFLP analysis. J. Exp. Mar. Biol. Ecol. 226, 279-291. doi: 10.1016/S0022-0981(97)00250-5.

Boursot P., Bonhomme F., 1986. Génétique et evolution du génome mitochondrial des métazoaires. Gen. Select. Evol., 18: 73-98.

Bridgham J.T., Wilder J.A., Hollocher H., Johnson A.L., 2003. All in the family: evolutionary and functional relationship among death receptors. Cell Death Differ., 10: 19-25. doi: 10.1038/sj.cdd.4401174.

Bucklin A., Steinke D., Blanco-Bercial L., 2011. DNA barcoding of marine Metazoa. Annu. Rev. Mar. Sci., 3: 471–508. doi: 10.1146/annurev-marine-120308-080950.

Burge C.A., Judah L.R., Conquest L.L., Griffin F.J., Cheney D.P., Suhrbier A., Vadopalas B., Olin P.G., Renault T., Friedman C.S., 2007. Summer seed mortality of the Pacific oyster, *Crassostrea gigas* Thunberg grown in Tomales Bay, California, USA: the influence of oyster stock, planting time, pathogens, and environmental stressors. J. Shellfish Res., 26: 163-172.

Burioli E.A.V., Houssin M., Varello K., Gorla A., Fioravanti M.L., Prearo M., 2015. Isolamento di un membro della famiglia flavobacteriaceae in ostriche concave *Crassostrea gigas* durante un evento di mortalità nella laguna di san teodoro (OT). Atti del XXI Convegno Nazionale S.I.P.I. Società Italiana Patologia Ittica, 8-9 OTTOBRE 2015, Chioggia (VE).

Burioli E.A.V., Prearo M., Riina M.V., Bona M.C., Fioravanti M.L., Arcangeli G., Houssin M., 2016. Ostreid herpesvirus type 1 genomic diversity in wild populations of Pacific oyster *Crassostrea gigas* from Italian coasts. J. Invertebr. Pathol., 137: 71-83. doi: 10.1016/j.jip.2016.05.004.

Burton T.L., Husband B.C., 2000. Fitness differences among diploids, tetraploids, and their triploid progeny in *Chamerion angustifolium*: mechanisms of inviability and implications for polyploid evolution. Evolution, 54(4): 1182-1191. doi: 10.1111/j.0014.3820.2000.tb00553.x.

Cagliani R., Forni D., Tresoldi C., Pozzoli U., Filippi G., Rainone V. *et al*., 2014. RIG-I-like receptors evolved adaptively in mammals, with parallel evolution at LGP2 and RIG-I. J. Mol. Biol., 426: 1351-1365. doi: 10.1016/j.jmb.2013.10.040.

Cai M.S., Li M., Zheng C.F., 2012. Herpesviral infection and Toll-likebreceptor 2. Protein & cell, 3 : 590-601.doi: 10.1007/s13238-012-2059-9.

Carballal M.J., Villalba A., Iglesias D., Hine P.M., 2003. Virus like particles associated with large foci of heavy hemocytic infiltration in cockles *Cerastoderma edule* from Galicia (NW Spain). J. Invertebr. Pathol., 84: 234-237. doi: 10.1016/j.jip.2003.11.002.

Chagot D., Boulo V., Hervio D., Mialhe E., Bachère E., Mourton C., Grizel H., 1992. Interactions between *Bonamia ostreae* (Protozoa: *Ascetospora*) and haemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca: *Bivalvia*): entry mechanisms. J. Invertebr. Pathol., 59: 241-249. doi: 10.1016/0022-2011(92)90127-P.

Chang P.H., Kuo S.T., Lai S.H., Yang H.S., Ting Y.Y., Hsu C.L., Chen H.C., 2005. Herpes-like virus infection causing mortality of cultured abalone *Haliotis diversicolor* supertaxa in Taiwan. Dis. Aquat. Org., 65: 23-27. doi : 10.3354/dao065023.

Chávez-Villalba J., Barret J., Mingant C., Cochard J.C., Le Pennec M., 2003. Influence of timing of broodstock collection on conditioning, oocyte production, and larval rearing of the oyster *Crassostrea gigas* (Thunberg) at six production sites in France. J. Shellfish Res., 22:465-474.

Chee M.S., Bankier A.T., Beck S., Bohni R., Brown C.M., Cerny R., Horsnell T., Hutchison T.A., Kouzarides I.T., Martignetti J.A., Preddie E., Satchwell S.C., Tomlinson P.,

Weston KM, Barrel G.,1990. Analysis of the Protein-Coding Content of the Sequence of Human Cytomegalovirus Strain AD169. In: McDougall J.K. (eds) Cytomegaloviruses. Current Topics in Microbiology and Immunology, vol 154. Springer, Berlin, Heidelberg.

Chen Z.J., Ni Z., 2006. Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. Bioessays, 28: 240-252. doi: 10.1002/bies.20374.

Cheng T., 1981. Invertebrate blood cells. Acad. Press Lond.

Cochennec-Laureau N., Baud J.P., Bedier E., Boudry P., Huvet A., Nicolas J.L., Pépin J.F., Petton B., 2009. "Journées Surmotalités des huitres creuses, *Crassostrea gigas*" du programme P7 "Aquaculture Durable" des 8-9 décembre 2009. htpp://sd2.archivehost.com/membres/up/214153133033562310/IFREMER_8_et_9_decembre_2009.pdf.

Coe W.R., 1936. Environment and sex in the oviparous oyster *Ostrea virginica*. Biol. Bull., 71(2): 353-359.

Colas, P. Dubé, F., 1998. Meiotic maturation in mollusc oocytes. Sem. Cell Dev. Biol., 9 : 539-548. doi : 10.1006/scdb.1998.0248.

Comps M., 1970a. Observations sur les causes d'une mortalité anormale des huîtres plates (*Ostrea edulis* L.) dans le bassin de Marennes. Rev. Trav. Inst. Pêches Marit., 34(3):317-326.

Comps M., 1970b. La maladie des branchies chez les huîtres du genre *Crassostrea*. Caractéristiques et évolution des altérations, processus de cicatrisation. Rev. Trav. Inst. Pêches Marit., 34: 24-43.

Comps M., 1983. Recherches histologiques et cytologiques sur les infections intracellulaires des Mollusques bivalves marins. Thèse Doctorat d'Etat en Sciences Naturelles, Université de Montpellier, 128 pp.

Comps M., Bonami,J.R., Vago C., Campillo A., 1976. Une virose de l'huître portugaise (*Crassostrea angulata* Lmk). Comptes Rendus Académie Sci. Paris 1991–1196.

Comps M., Cochennec N., 1993. A herpes-like virus from the European oyster *Ostrea edulis* L. J. Inverteb. Pathol., 62: 201-203. doi : 10.1006/jipa.1993.1098.

Comps M., Duthoit J.L., 1979. Infections virales chez les huîtres *Crassostrea angulata* (Lmk) and *C. gigas* (Th.). Haliotis, 8: 301-308.

Comps M., Herbaut C., Fougerousse A., 1999. Virus-like particles in pearl oyster *Pinctada margaritifera*. Bull. Eur. Assoc. Fish Pathol., 19: 85-88.

Comps M., Tige G., Grizel H., 1980. Etude ultrastructurale d'un protiste de l'huître *Ostrea edulis* L.. C.R. Acad. Sc. Paris, Série D, 290: 383-384.

Corbeil S., Faury N., Segarra A., Renault T., 2015. Development of an *in situ* hybridisation assay for the detection of Ostreid herpesvirus 1 mRNAs in the Pacific oyster, *Crassostrea gigas*. J. Virol. Methods, 211: 43-50. doi: 10.1097/01.PAT.0000426854.40554.fa.

Corbeil S., Williams L.M., Bergfeld J., Crane M.S.J., 2012. Abalone herpes virus stability in sea water and susceptibility to chemical disinfectants. Aquaculture, 326-329: 20-26. doi : 10.1016/j.aquaculture.2011.11.031.

Corporeau C., Tamayo D., Pernet F., Quéré C., Madec S., 2014. Proteomic signatures of the oysters metabolic response to herpesvirus OsHV-1 µVar infection. J. Proteomics, 109: 176-187. doi: 10.1016/j.prot.2014.06.030.

Cory S., Adams J.M., 2002. The Bcl-2 family: regulators of the cellular life -or-death switch. Nat. Rev. Cancer, 2: 647-656. doi: 10.1038/nrc883.

Costil, K., Royer, J., Ropert, M., Soletchnik, P., Mathieu, M., 2005. Spatio-temporal variations in biological performances and summer mortality of the Pacific oyster *Crassostrea gigas* in Normandy (France). Helgol. Mar. Res. 59, 286–300. doi:10.1007/s10152-005-0004-5.

Crook N.E., Clem R.J., Miller L.K., 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J. Virol., 67: 2168-2174. PMCID: PMC240327.

Da Silva P.M., Renault T., Fuentes J., Villalba A., 2008. Herpesvirus infection in European flat oysters *Ostrea edulis* obtained from brood stocks of various geographic origins and grown in Galicia (NW Spain). Dis. Aquat. Org., 78: 181-188. doi: 10.3354/dao01874.

Davison A.J., 2002. Evolution of the herpesviruses. Vet. Microbiol., 86: 69-88. doi: 10.1016/S0378-1135(01)00492-8.

Davison A.J., Eberle R., Ehlers B., Hayward G.S., McGeoch D.J., Minson A.C., Pellett P.E., Roizman B., Studdert M.J., Thiry E., 2009. The order *Herpesvirales*. Arch. Virol. 154 (19), 171–177. doi: 10.1007/s00705-008-0278-4.

Davison, A.J., Trus, B.L., Cheng, N., Steven, A.C., Watson, M.S., Cunningham, C., Le Deuff, R.M., Renault, T., 2005. A novel class of herpesvirus with bivalve hosts. J. Gen. Virol. 86 (1), 41–53. doi: 10.1099/vir.0.80382-0.

De Lorgeril J., Zenagui R., Rosa R.D., Piquemal D., Bachère E., 2011. Whole transcriptome profiling of successful immune response to Vibrio infections in the oyster *Crassostrea gigas* by digital gene expression analysis. PlosOne, 6, e23142. Doi: 10.1371/journal.pone.0023142.

Defer D., 2010. Recherche d'activités antimicrobiennes chez des mollusques marins. Purification et caractérisation partielle de peptides antimicrobiens isolés à partir de l'hémolymphe de *Crassostrea gigas* et de bactéries associées. Université de Bretagne Sud.

Dégremont L., 2011. Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture, 299: 44-50. doi: 10.1016/j.aquaculture.2011.04.029.

Dégremont L., Garcia C., Allen S.K.Jr., 2015. Genetic improvement for disease resistance in oysters: A review. J. Inv. Pathol., 131: 226-241. doi: 10.1016/j.jip.2015.05.010.

Deveraux Q.I., Reed J.C., 1999. IAP family proteins-suppressors of apoptosis. Genes Dev., 13: 239-252.

Dundon W.G., Arzul I., Omnes E., Robert M., Magnabosco C., Zambon M., Gennari L., Toffan A., Terregino C., Capua I., Arcangeli G., 2011. Detection of Type 1 Ostreid Herpes variant (OsHV-1 µVar) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. Aquaculture, 314(1-4): 49-52. doi: 10.1016/j.aquaculture.2011.02.005.

EFSA, European Food Safety Agency, 2014a. Scientific Opinion on health benefits of seafood (fish and shellfish) consumption in relation to health risks associated with exposure to methylmercury. EFSA J., 12(7): 3761. doi: 10.2903/j.efsa.2014.3761.

Elmore S. 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol., 35: 495-516. doi: 10.1080/01926230701320337.

Elston R., Wilkinson M.T., 1985. Pathology, management and diagnosis of oyster velar virus disease (OVVD). Aquaculture, 48: 189-210. doi: 10.1016/0044-8486(85)90124-3.

Elston R.A., 1979. Virus-like particles associated with lesions in larval pacific oysters (*Crassostrea gigas*). J. Invert. Pathol., 33(1): 71-74. doi: 10.1016/0022-2011(79)90132-0.

Elston R.A., Hasegawa H., Humphrey K.L., Polyak I.K., Hase C.C., 2008. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Dis. Aquat. Organ., 82: 119. doi: 103354/dao01982.

Elston R.A., Moore J.D., Brooks K., 1992. Disseminated neoplasia of bivalve molluscs. Rev. Aquat. Sci., 6: 405-466. doi: 10.1007/s11745-014-3917-4.

Engelsma M.Y., Roozenburg I., Joly J.P., 2008. First isolation of *Nocardia crassostreae* from Pacific oyster *Crassostrea gigas* in Europe. Dis. Aquat. Org., 80: 229-234. doi : 10.3354/dao01938.

Enríquez-Díaz M., Pouvreau S., Chávez-Villalba J., Le Pennec M., 2009. Gametogenesis, reproductive investment, and spawning behavior of the Pacific giant oyster *Crassostrea gigas*: evidence of an environment-dependent strategy. Aquacult. Int., 17(5): 491-506. doi : 10.1007/s10499-008-9219-1.

Estévez-Calvar N., Romero A., Figueras A., Novoa B., 2013. Genes of the mitochondrial apoptotic pathway in *Mytilus galloprovincialis*. PlosOne 8, e61502. doi: 10.1371/journal.pone.0061502.

Evans O., Paul-Pont I., Hick P., Whittington R., 2014. A simple centrifugation method for improving the detection of Ostreid herpesvirus-1 (OsHV-1) in natural seawater samples with an assessment of the potential for particulate attachment. J. Virol. Methods, 210: 59-66. doi: 10.1016/j.jviromet.2014.09.023.

FAO, Food and Agriculture Organization of the United Nations, 2014. Fisheries and Aquaculture Department, Dataset Global Aquaculture Production. On-line Query.

FAO/WHO, 2010. Report of the joint FAO/WHO expert consultation on the risks and benefits of fish consumption, Rome, 25–29 January 2010 FAO Fisheries and Aquaculture Report No. 978 FIPM/R978(En).

Farley C.A., Banfield W.G., Kasnic Jr. G., Foster W.S., 1972. Oyster herpes-type virus. Science, 178: 759–760. doi: 10.1126/science.178.4062.759.

Felsenstein J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-791. doi: 10.2307/2408678.

Feng S.Y., 1988. Cellular defense mechanisms of oysters and mussels. Am. Fish Soc. Spec. Publ., 18: 153–168.

Fields B.N., Knipe D.M., Howley P.M., 2002. Fields Virology, 4th edn. Lippincott Williams & Wilkins: Philadelphia, PA.

Franc A., 1960. Classe des bivalves. In P.P. Grasse ed., Traité de Zoologie, Anatomie, Systématique, Biologie, tome V, fascicule 2, pp.1845-2133. Masson et Cie, Paris.

Friedman C.S., Beattie J.H., Elston R.A., Hedrick R.P., 1991. Investigation of the relationship between the presence of a Gram-positive bacterial infection and summer mortality of the Pacific oyster, *Crassostrea gigas* Thunberg. Aquaculture, 94: 1-15.

Friedman C.S., Estes R.M., Stokes N.A, Burges C.A., Hargove J.S., Barber B.J, Elston R.A., Burreson E.M., Reece K.S., 2005. Herpes virus in juvenile Pacific oysters Crassostrea gigas from Tomales Bay, California, coincides with summer mortality episodes. Dis. Aquat. Org., 63: 33-41. doi: 10.3354/dao063033.

Funes V., Alhama J., Navas J.I., López-Barea J., Peinado J., 2006. Ecotoxicological effects of metal pollution in two mollusc species from the Spanish South Atlantic littoral. Environ. Pollut., 139(2): 214-223. doi: 10.1016/j.env.pol.2005.05.016.

Furlong D., Swift H., Roizman B., 1972. Arrangement of herpesvirus deoxyribonucleic acid in the core. J. Virol., 10: 1071-1074.

Gabbott P.A., 1983. Developmental and seasonal metabolic activities in marine molluscs. In : The Mollusca, Vol. 2, Environmental Biochemistry and Physiology, P.W. Hochachka ed., pp. 165-217, Academic Press, New York.

Gaffney P.M., Allen S.K. Jr., 1993. Hybridization among *Crassostrea* species: a review. Aquaculture, 116: 1-13.

Galtsoff, PS. 1964. The American oyster, *Crassostrea virginica* Gmelin. Fishery Bulletin, Vol. 64. United States Government Printing Office, Washington, D. C., pp. 480.

Garcia-Blanco M.A., Cullen B.R., 1991. Molecular basis of latency in pathogenic human viruses. Science, 254 : 815–820.

Garnier M., Labreuche Y., Garcia C., Robert M., Nicolas J.-L., 2007. Evidence for the Involvement of Pathogenic Bacteria in Summer Mortalities of the Pacific Oyster *Crassostrea gigas*. Microb. Ecol., 53, 187–196. doi:10.1007/s00248-006-9061-9.

Garnier M., Labreuche Y., Nicolas J.-L., 2008. Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. Syst. Appl. Microbiol. 31: 358–365. doi:10.1016/j.syapm.2008.06.003.

Ghisotti F., 1971. Molluschi del genere *Crassostrea* nell'alto Adriatico. In Conchiglie, Notiziario Mensile della Unione Malacologica Italiana, 7-8: 113-124.

Gibson W., 1996. Structure and assembly of the virion. Intervirology, 39: 389–400.

Gittenberger A., Voorbergen-Laarman M.A., Engelsma M.Y., 2016. Ostreid herpesvirus OsHV-1 µVar in Pacific oysters *Crassostrea gigas* (Thunberg, 1793) of the Wadden Sea, a UNESCO world heritage site. J. Fish Dis., 39(1): 105-109. doi: 10.1111/jfd.12332.

Gong N., Yang H., Zhang G., Landau BJ., Guo X., 2004. Chromosome inheritance in triploid Pacific oyster *Crassostrea gigas* Thunberg. Heredity, 93: 408-415.

Gonzalez M., Gueguen Y., Desserre G., de Lorgeril J., Romestand B., Bachère E., 2007a. Molecular characterization of two isoforms of defensin from haemocytes of the oyster *Crassostrea gigas*. Dev. Comp. Immunol., 31: 332-339. doi: 10.1016/j.dci.2006.07.006.

Gonzalez M., Gueguen Y., Destoumieux-Garzón D., Romestand B., Fievet J., Pugnière M., et al., 2007b. Evidence of a bactericidal permeability increasing protein in an invertebrate, the *Crassostrea gigas* Cg-BPI. Proc. Natl. Acad. Sci. U.S.A., 104: 17759-17764. doi: 10.1073/pnas.0702281104.

Goulletquer P., Heral M., 1997. Marine molluscan production trends in France: from fisheries to aquaculture. In U.S. Dep. Commer., NOAA Technical Report. NMFS 129, 137-164.

Goulletquer P., Soletchnik P., Le Moine O., Razet D., Geairon P., Faury N., 1998. Summer mortality of the Pacific cupped oyster *Crassostrea gigas* in the Bay of Marennes-Oleron (France). Presented at the CIEM Conseil International pour l'Exploration de la mer.

Grant V., 1981. Plant Speciation .New York: Columbia Univ. Press. 2nd ed.

Green T.J, Montagnani C., 2013. Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid herpesvirus (OsHV-1 µVar). Fish Shellfish Immunol., 35: 382-388.

Green T.J., Benkendorff K., Robinson N., Raftos D., Speck P., 2014. Anti-viral gene induction is absent upon secondary challenge with double-stranded RNA in the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol., 36: 151-157.

Green T.J., Speck P., Geng L., Raftos D., Beard M.R., Helbig K.J., 2015. Oyster viperin retains direct antiviral activity and its transcription occurs via a signalling pathway involving a heat-stable haemolymph protein. J. Gen. Virol., 96: 3587-3597. doi: 10.1099/jgv.0.000300.

Gregory T.R., Mable, B.K., 2005. Polyploidy in animals. In The Evolution of the Genome, T.R. Gregory, ed. (San Diego: Elsevier), pp. 427–517.

Grijalva-Chon J.M., Castro-Longoria R., Ramos-Paredes J., Enríquez-Espinoza T.L., Mendoza-Cano F., 2013. Detection of a new OsHV-1 DNA strain in the healthy Pacific oyster, *Crassostrea gigas* (Thunberg) from the Gulf of California. J. Fish Dis., 36: 965-968. doi: 10.1111/jfd.12028.

Grinde B., 2013. Herpesviruses: latency and reactivation -viral strategies and host response. J. Oral Microbiol., 5: 22766. doi: 10.3402/jom.v5i0.22766.

Grizel H., 1985. Etude des récentes épizooties de l'huître plate *Ostrea edulis* Linné et de leur impact sur l'ostréiculture bretonne. Thèse Doctorat d'Etat en Sciences Naturelles, Université de Montpellier, 145 pp.

Grizel H., Comps M., Bon Ami J.R., Cousserans F., Duthoit J.L., Le Pennec M.A., 1974. Recherche de l'agent de la maladie de la glande digestive de *Ostrea edulis*, Linné. Bull. Inst. Pêches. Marit, 240: 7-30.

Gubser C., Hué S., Kellam P., Smith G.L., 2004. Poxvirus genomes: a phylogenetic analysis. J. Gen. Virol., 85: 105-117.

Gueguen Y., Cadoret J.P., Flament D., Barreau-Roumiguière C., Girardot A.L., Garnier J., Hoareau A., Bachère, E., Escoubas, J.-M., 2003. Immune gene discovery by expressed sequence tags generated from haemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. Gene, 303: 139–145. doi:10.1016/S0378-1119(02)01149-6.

Gueguen Y., Herpin A., Aumelas A., Garnier J., Fievet J., Escoubas J.M. et al., 2006. Characterization of a defensin from the oyster *Crassostrea gigas*. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. J. Biol. Chem., 281: 313-323. doi: 10.1074/jbc.M510850200.

Guo X., Allen S.K. Jr., 1994. Reproductive Potential and Genetics of Triploid Pacific Oysters, *Crassostrea gigas* (Thunberg). Biol. Bull., 187:3, 309-318.

Guo X., Cooper K., Hershberger WK., Chew KK., 1992. Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*: I. Ploidy of resultant embryos. Biol Bull., 183: 381-386.

Guo X., DeBrosse G.A., Alle S.K.Jr., 1996. All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture, 142: 149-161.

Guo X., He Y., Zhang L., Lelong C., Jouaux A., 2015. Immune and stress responses in oysters with insights on adaptation. Fish Shellfish Immunol., 46(1): 107-119.

Hasegawa H., Gharaibeh D.N., Lind E.J., Häse C.C., 2009. Virulence of metalloproteases produced by Vibrio species on Pacific oyster *Crassostrea gigas* larvae. Dis. Aquat. Organ., 85(2): 123-131. doi: 10.3354/dao02070.

Hayward G.S., Jacob R.J., Wadsworth S.C, Roizman B., 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc. Natl. Acad. Sci. USA: 72: 4243-4247.

He X., Zhang Y., Yu Z., 2011. An Mpeg (macrophage expressed gene) from the Pacific oyster Crassostrea gigas: molecular characterization and gene expression. Fish Shellfish Immunol., 30: 870-876. doi: 10.1016/j.fsi.2011.01.009.

He Y., Jouaux A., Ford S.E., Lelong C., Sourdaine P., Mathieu M., Guo X., 2015. Transcriptome analysis reveals strong and complex antiviral response in a mollusc. Fish Shellfish Immunol., 46(1): 131-144. doi: 10.1016/j.fsi.2015.05.023.

Hegarty M., Hiscock S. 2007. Polyploidy: doubling up for evolutionary success. Curr. Biol., 17: 927-929.

Helm M.M., Bourne N., Lovatelli A., 2004. Hatchery culture of bivalves. Technical paper. Rome, Italy, FAO.

Herrbach B., 1971. Sur une affection parasitaire de la glande digestive de l'huître plate *Ostrea edulis* Linné. Rev. Trav. Inst. Pêches Marit., 35(l): 79-87.

Heude-Berthelin C., Laisney J., Espinosa J., Martin O., Hernandez G., Mathieu M., Kellner K., 2001. Storage and reproductive strategy in *Crassostrea gigas* from two different growing areas (Normandy and the Atlantic coast, France). J. Invertebr. Reprod. D., 40(1): 79-86. doi: 10.1080/07924259.2001.9652500.

Hick P., Evans O., Looi R., English C., Whittington R.J., 2016. Stability of Ostreid herpesvirus-1 (OsHV-1) ans assessment of disinfection of seawater and oyster tissues using a bioassay. Aquaculture, 450: 412-421.

Hine P., 1999. The inter-relationships of bivalve haemocytes. Fish Shellfish Immunol., 9: 367-385. doi: 10.1006/fsim.1998.0205.

Hine P.M., Thorne T., 1997. Replication of herpes-like viruses in haemocytes of adult flat oyster Ostrea angasi: an ultrastructural study. Dis. Aquat. Org., 29: 189-196.

Hine P.M., Wesney B., Besant P., 1998. Replication of herpes-like virus in larvae of the flat oyster *Tiostrea chilensis* at ambient temperatures. Dis. Aquqt. Org., 32: 161-171.

Hine, P.M., Wesney, B., Hay, B.E., 1992. Herpes virus associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*. Dis. Aquat. Org.,12, 135–142.

Hirano M., Das S., Guo P., Cooper M.D., 2011. The Evolution of Adaptive Immunity in Vertebrates, Chap. 4. Adv. Immunol., 109: 125–157.

Hoffmann J.A., Kafatos F.C., Janeway C.A.Jr., Ezekowitz R.A.B., 1999. Phylogenetic Perspectives in Innate Immunity. Science, 284(5418): 1313-8.

Honess R.W., Roizman B., 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14:8–19

House M.L., Kim C.H., Reno P.W., 1998. Soft shell clams *Mya arenaria* with disseminated neoplasia demonstrate reverse transcriptase activity. Dis. Aquat. Org., 34: 187-192.

Huff J.L., Barry P.A., 2003. B-virus (Cercopithecine herpesvirus 1) Infections in humans and macaques: potential for zoonotic disease. Emerg. Infect. Dis., 9(2): 245-250. doi: 10.3201/eid0902.020272.

Huvet A., Balabaud K., Bierne N., Boudry P., 2001. Microsatellite analysis of 6-hour-old embryos reveals no preferential intra-specific fertilization between cupped oysters *Crassostrea gigas* and *Crassostrea angulata*. Mar. Biotechnol., 3: 448-453.

Huvet A., Boudry P., Ohresser M., Delsert C., Bonhomme F., 2000a. Variable microsatellites in the Pacific cupped oyster *Crassostrea gigas* and other cupped oyster species ; Anim. Gen., 37: 71-72.

Huvet A., Fabioux C., McCombie H., Lapègue S., Boudry P., 2004. Natural hybridization between genetically differentiated populations of *Crassostrea gigas and C. angulata* highlighted by sequence variation in flanking regions of a microsatellite locus. Mar. Ecol. Prog. Ser., 272: 141-152.

Huvet A., Gérard A., Ledu C., Phélipot P., Heurtebise S., Boudry P., 2002. Is fertility of hybrids enough to conclude that the two oysters *Crassostrea gigas* and *Crassostrea angulata* are the same species. Aquat. Living Resour., 15: 45-52.

Huvet A., Lapègue S., Magoulas A., Boudry B., 2000b. Mitochondrial and nuclear DNA phylogeography of *Crassostrea angulata*, the Portuguese oyster endangered in Europe. Conserv. Gen., 1: 251-262.

Hwang J.Y., Park J.J., Yu H.J., Hur Y.B., Arzul I., Couraleau Y., Park M.A., 2013. Ostreid herpesvirus 1 in Pacific oyster *Crassostrea gigas* larvae (Thunberg) in Korea. J. Fish Dis., 36(11): 369-372. doi: 10.1111/jfd.12093.

Imai T., Numachi K., Oizumi J., Sato S., 1965. Studies on the Mass Mortality of the Oyster in Matsushima Bay II. Search for the Cause of Mass Mortality and the Possibility to Prevent it by Transplantation Experiment. Bull. Tohoku Natl. Fish. Res. Inst. Shiogama, 27-38.

Inoue N., Dambaugh T.R., Rapp J.C., Pellett P.E., 1994. Alphaherpesvirus origin-binding protein homolog encoded by human herpesvirus 6B, a betaherpesvirus, binds to nucleotide sequences that are similar to ori regions of alphaherpesviruses. J. Virol., 68: 4126-4136.

Jakob N.J., Müller K., Bahr U., Darai G., 2001. Analysis of the first complete DNA sequence of an invertebrate iridovirus: coding strategy of the genome of *Chilo* iridescent virus. Virology, 286: 182-196. doi:10.1006/viro.2001.0963.

Jayan G.C., Casey J.L., 2002. Incresed RNA editing and inhibition of hepatitis delta virus replication by high level expression of ADAR1 and ADAR2. J. Virol., 76: 3819-3827.

Jenkins C., Hick P., Gabor M., Spiers Z., Fell S.A., Gu X., Read A., Go J., Dove M., O'Connor W., Kirkland P.D., Frances J., 2013. Identification and characterisation of an

ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* in Pacific oysters in Australia. Dis. Aquat. Organ. 105(2): 109-126. doi: 10.3354/dao02623.

Jouaux A., 2010. Gestion de l'allocation d'énergie à la croissance ou à la reproduction chez l'huître creuse *Crassostrea gigas*: implication d'une régulation par les insulin-like. Université de Caen Basse-Normandie. Thèse.

Jouaux A., Heude-Berthelin C., Sourdaine P., Mathieu M., Kellner K., 2010. Gametogenic stages in triploid oysters *Crassotrea gigas*: irregular locking of gonial proliferation and subsequent reproductive effort. J. Exp. Mar. Biol. Ecol., 395: 162-170. doi: 10.1016/j.jembe.2010.08.030.

Jouaux A., Lafont M., Blin J.L., Houssin M., Mathieu M., Lelong C., 2013. Physiological change under OsHV-1 contamination in Pacific oyster *Crassostrea gigas* through massive mortality events on fields. BMC genomics, 14: 590. doi:

Kawasaki H., Iwamuro S., 2008. Potential role of histones in host defense as antimicrobial agents. Infect. Disord. Drug Targets, 8: 195-205.

Keeling S.E., Brosnahan C.L., Williams R., Gias E., Hannah M., Bueno R., McDonald W.L., Johnston C., 2014. New Zealand juvenile oyster mortality associated with Ostreid herpesvirus 1 – an opportunistic longitudinal study. Dis. Aquat. Organ., 109: 231-239. doi: 10.3354/dao02735.

Kelly B.J., Fraefel C., Cunningham A.L., Diefenbach R.J., 2009. Functional roles of the tegument proteins of herpes simplex virus type 1. Virus Res., 145(2): 173-186.

Kennedy A.V., Battle H.I., 1964. Cyclic changes in the gonad of the American oyster, *Crassostrea virginica* (Gmelin). Can. J. Zool., 42:305-321.

Kieff E., Rickinson A.B., 2001. Epstein-Barr virus and its replication, p. 2511-2573. In D. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.

Kumar S., Stecher G., and Tamura K., 2015. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets.Molecular Biology and Evolution (submitted).

La Peyre J.F., Yarnall H.A., Faisal M., 1996. Contribution of *Perkinsus marinus* extracellular products in the infection of Eastern Oysters (*Crassostrea virginica*). J. Invertebr. Pathol., 68: 312-313.

Labreuche Y., Lambert C., Soudant P., Boulo V., Huvet A. & Nicolas J.L., 2006b. Cellular and molecular haemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. Microbes Infect., 8: 2715-2724.

Labreuche Y., Soudant P., Goncalves M., Lambert C., Nicolas J.L. 2006a. Effects of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on lethality and cellular immune responses of the oyster *Crassostrea gigas.* Dev. Comp. Immunol., 30: 367-379.

Lacoste A., Jalabert F., Malham S., Cueff A., Gelebart F., Cordevant C., Lange M., Poulet S.A., 2001. A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). Dis. Aquat. Organ., 46: 139-145.

Lafferty K.D., Harvell C.D., Conrad J.M., Friedman C.S., Kent M.L., Kuris A.M., Powell E.N., Rondeau D., Saksida S.M., 2015. Infectious Diseases Affect Marine Fisheries and

Aquaculture Economics. Ann. Rev. Mar. Sci., 7: 471-496. doi: 10.1146/annurev-marine-010814-015646.

Lafferty K.D., Porter J.W., Ford S.E., 2004. Are diseases increasing in the ocean? Annu. Rev. Evol. Syst., 35: 31-54.

Lambert C., Soudant P., Choquet G., Paillard C., 2003. Measurement of *Crassostrea gigas* haemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish Shellfish Immunol., 15: 225-240.

Langmead B., Salzberg S., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods, 9: 357–359. doi:10.1038/nmeth.1923.

Le Dantec J. , 1968 Ecologie et reproduction de l'huitre portugaise *Crassostrea angulata* dans le bassin d'Arcachon et sur la rive gauche de la Gironde. Revue des Travaux de l'Institut des Pêches Maritimes, 32: 237-362.

Le Deuff R.M., Nicolas J.L., Renault T., Cochennec N., 1994. Experimental transmission of a herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas.* Bull. Eur. Fish Pathol., 14(2): 69-71.

Le Deuff R.M., Nicolas J.L., Renault T., Cochennec N., 1994. Experimental transmission of a herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. Bull. Eur. Ass. Fish Pathol., 14(2): 69-72.

Le Deuff R.M., Renault T., 1999. Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. J. Gen. Virol. 80, 1317–1322.

Le Deuff R.M., Renault T., Cochennec N., 1995. Antobodies specific for channel catfish virus cross-react with Pacific oyster, *Crassostrea gigas*, herpes-like virus. Vet. Res., 26. 526-529.

Le Roux F., Gay M., Lambert C., Waechter M., Poubalanne S., Chollet B., Nicolas J.-L., Berthe, F., 2002. Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. Aquat. Living Resour.,15: 251-258.

Lemire A., Goudenege D., Versigny T., Petton B., Calteau A., Labreuche Y., Le Roux F., 2015. Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. ISME J., 9(7): 1523-1531. doi: 10/1038/ismej.2014.233.

Leulier F., Lemaitre B., 2008. Toll-like recepttors – taking an evolutionary approach. Nat. Rev. Gen., 9 : 165-178.

Li L., Qiua L., Song L., Song X., Zhao J., Wang L., *et al.*, 2009. First molluscan TNFR homologue in Zhikong scallop: molecular characterization and expression analysis. Fish Immunol., 27: 625-632.

Li Q., Spriggs M.K., Kovats S., Turk S.M., Comeau M.R., Nepom B., Hutt-Fletcher L.M., 1997. Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. J. Virol., 71: 4657-4662.

Lipart C., Renault T., 2002. Herpes-like virus detection in *Crassostrea gigas* spat using DIG-labelled probes. J. Virol. Methods, 101: 1–10.

Loker E.S., Adema C.M., Zhang S.M., Kepler T.B., 2004. Invertebrate immune systems – not homogeneous, not simple, not well understood. Immunol. Rev:, 198: 10-24.

Lokmer A., Wegner K.M., 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. ISME J., 9: 670-682.

López JR, Piñeiro-Vidal M, García-Lamas N, de la Herran R, Navas JI, Hachero-Cruzado I & Santos Y., 2010. First isolation of Tenacibaculum soleae from diseased cultured wedge sole, *Dicologoglossa cuneate* (Moreau), and brill, *Scophthalmus rhombus* (L.). J Fish Dis 33 : 273–278.

Loret S., Guay G., Lippe R., 2008. Comprehensive characterization of extracellular herpes simplex virus type 1 virions. J. Virol. 82 (17): 8605–8618.

Lorteau C., Auffret M., Le Bris H., 1995. The immunodefense system of bivalves. 1. Stucture and working [haemocytes]. Recl. Med. Veterinaire 171.

Lyman M.G., Enquist L.W., 2009. Herpesvirus interactions with the host cytoskeleton. J. Virol., 83 : 2058-2066.

Lynch S.A., Carlsson J., Reilly A.O., Cotter O., Culloty S.C., 2012. A previously undescribed Ostreid herpes virus 1 (OsHV-1) genotype detected in the Pacific oyster, *Crassostrea gigas*, in Ireland. Parasitology, 139(12): 1526-1532. doi: 10.1017/S0031182012000881.

Lyons M.M, Ward J.E., 2005. Lethal marine snow: pathogen of bivalve mollusc concealed in marine aggregates. Limnol. Oceanogr., 50: 1983-1988.

Mable B.K., 2004. Why polyploidy is rarer in animals than in plants: myths and mechanisms. Biol. J. Linn. Soc., 82 (4), 453–466.

Mann R., Burreson E., Baker P., 1991. The decline of the Virginia oyster fishery in Chesapeake Bay: considerations for the introduction of a non-endemic species, *Crassostrea gigas* (Thunberg, 1793). J. Shellfish Res., 10: 379-388.

Manzoni, P., 2010. Grande enciclopedia illustrata dei Crostacei dei Molluschi e dei Ricci di mare. Eurofishmarket 3 dicembre 2010.

Marceau F., 1904a. Sur les fonctions respectives des deux parties des muscles adducteurs chez les lamellibranches. C. R. Hebd. Acad. Sci., tome 138, pp. 1343-1345.

Marceau F., 1904b. Sur le mécanisme de la contraction des fibres musculaires lisses dites à double striation oblique ou à fibrilles spiralées et en particulier de celles des muscles adducteurs des Lamellibranches. C. R. Hebd. Acad. Sci., tome 139, pp.70-73.

Marino F., Bambir S., Lanteri G., Rapisarda G., Macrì B., 2005. Contributo alla conoscenza di alcune patologie dell'abalone in allevamento sperimentale. Ittiopatologia, 3: 217-222.

Marshall B., Gofas S., 2015. *Crassostrea gigas*. In: MolluscaBase (2016). Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=140656 on 2017-01-03.

Martenot C., Denechère L., Hubert P., Metayer L., Oden E., Trancart S., Travaillé E., Houssin M., 2015. Virulence of Ostreid herpesvirus 1 µVar in sea water at 16°C and 25°C. Aquaculture, 439: 1-6. doi: 10.1016/j.aquaculture.2015.01.012.

Martenot C., Fourour S., Oden E., Jouaux A., Travaillé E., Malas J.P., Houssin M., 2012. Detection of the OsHV-1 µVar in the Pacific oyster *Crassostrea gigas* before 2008 in France and description of two new microvariants of the Ostreid Herpesvirus (OsHV-1). Aquaculture, 338-341: 293-296. doi: 10.1016/j.aquaculture.2011.12.030.

Martenot C., Oden E., Travaillé E., Malas J.P., Houssin M., 2011. Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. Virus Res., 160: 25-31. doi: 10.1016/j.virusres.2011.04.012.

Martenot C., Segarra A., Baillon L., Faury N., Houssin M., Renault T., 2016. *In situ* localization and tissue distribution of ostreid herpesvirus 1 proteins in infected Pacific

oyster, *Crassostrea gigas*. J. Invertebr. Pathol., 136: 124-135. doi: 10.106/j.jip.2016.04.002.

Martenot C., Travaillé E., Lethuillier O., Lelong C., Houssin H., 2013. Genome exploration of six variants of the Ostreid Herpesvirus 1 and characterization of large deletion in OsHV-1 µVar specimens. Virus Res., 178: 462-470. doi: 10.106/j.virusres.2013.08.006.

Martín-Gómez I., Villalba A., Carballal M.J., Abollo E., 2014. Molecular characterisation of TNF, AIF, dermatopontin and VAMP genes of the flat oyster *Ostrea edulis* an analysis of their modulation by diseases. Gene, 533: 208-217.

Matthews R.E.F., 1982. Classification and nomenclature of viruses. Chap. The families and Groups. Fourth Report of the International Committee on Taxonomy of Viruses. Intervirology, 17(1-3): 41-178.

Maurer D., Comps M., His E., 1986. Caractéristiques des mortalités estivales de l'huître *Crassostrea gigas* dans le bassin d'Arcachon. Haliotis, 309–317.

Mayr E. (1942). Systematics and the origin of species. Columbia University Press, New York.

McCallum H.I., Kuris A., Harvell C.D., Lafferty K.D., Smith G.W., Porter J. (2004). Does terrestrial epidemiology apply to marine systems? Trends Ecol. Evol., 19: 585-591. doi:10.1016/j.tree.2004.08.009.

McCombie H., Ledu C., Phelipot P., Lapègue S., Boudry P., 2005. A complementary method for production of tetraploid *Crassostrea gigas* using crosses between diploids and tetraploids. Mar. Biotechnol., 7(4): 318-330.

McFall-Ngai M., Hadfield M.G., Bosch T.C.G., Carey H.V., Domazet-Lošo T., Douglas A.E., Dubilier N., Eberl G., Fukami T., Gilbert S.F., Hentsche U., King N., Kjelleberg S., Knoll A.H., Kremer N., Mazmanian S.K., Metcalf J.L., Nealson K., Pierce N.E., Rawls J.F., Reid A., Ruby E.G., Rumpho M., Sanders J.G., Tautz D., Wernegreen J.J., 2013. Animals in a bacterial world, a new imperative for the life sciences. PNAS, 110(9): 3229–3236. doi: 10.1073/pnas.1218525110.

Menzel R.W., 1974. Portuguese and Japanese oysters are the same species. J; Fish Res. Board Can., 31: 453-546.

Metchnikov, E., 1884. Uber eine sprosspilzkrankheit der daphnien. Beitragzur lehre uber den kampf der phagocyten gegen krankheitserreger. Virchows Arch. 96, 177–195.

Minson, A.C., Davison, A., Eberle, R., Desrosiers, R.C., Fleckenstein, B., McGeoch, D.J., Pellet, P.E., Roizman, B., Studdert, M.J., 2000. Family *Herpesviridae*. In: Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses, Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 203–225.

Miyasaki T., Goto K., Kobayashi T., Kageyama T., Miyata M., 1999. Mass mortalities associated with a virus disease in Japanese pearl oysters *Pinctada fucata martensii*. Dis. Aquat. Org., 37: 1-12.

Mojika K.D.A., Brussaard C.D.P., 2014. Factors affecting virus dynamics and microbial host-virus interactions in marine environments. FEM Microbiol. Ecol., 89: 495-515. doi: 10.1111/1574-6941.12343.

Monroe W.E., 1989. Cinical signs associated with pseudorabies in dogs. J. Am. Vet. Med. Assoc., 195: 599-602.

Montagnani C., Kappler C., Reichhart J.M., Escoubas J.M., 2004. Cg-Rel, the first Rel/NF-κB homolog characterized in a mollusk, the Pacific oyster *Crassostrea gigas*. FEBS Lett., 561: 75-82.

Morley N.J., 2010) Interactive effects of infectious diseases and pollution in aquatic molluscs. Aquat. Toxicol., 96(1): 27-36. doi: 10.1016/j.aquatox.2009.09.017.

Morrissey T., McCleary S., Collins E., Henshilwood K., Cheslett D., 2015. An investigation of Ostreid Herpes Virus microvariants found in *Crassostrea gigas* oyster producing bays in Ireland. Aquaculture, 442: 86-92. doi: 10.1016/j.aquaculture.2015.02.026.

Mortensen S., Strand Å., Bodvin T., Alfjorden A., Skår C.K., Jelmert A., Aspán A., Saelemyr L., Naustvoll L.J., Albertsen J., 2016. Summer mortalities and detection of Ostreid herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and Norway. Dis. Aquat. Organ., 117(3): 171-176. doi: 10.3354/dao02944.

Mourton C., Boulo V., Chagot D., Hervio D., Bachère E., Mialhe E., Grizel H., 1992. Interactions between *Bonamia ostreae* (Protozoa: Ascetospora) and haemocytes of *Ostrea edulis a*nd *Crassostrea gigas* (Mollusca: Bivalvia): In vitro system establishment. J. Invertebr. Pathol., 59: 235-240.

Mulcahy M.F., Roch P., 2009. Diseases and Pathology of Aquatic Organisms. Fisheries and Aquaculture. Vol. IV. Encyclopedia of Life Support Systems (EOLSS). Ed. P. Safran.

Mydlarz L.D., Jones L.E., Harvell C.D., 2006. Innate immunity environmental drivers and disease ecology of marine and freshwater invertebrates. Annu. Rev. Ecol. Evol. Syst., 37: 251-288.

Nasfi H., Travers M.A., de Lorgeril, J., Habib, C., Sannie, T., Sorieul, L., Gerard, J., Avarre, J.C., Haffner, P., Tourbiez, D., Renault, T., Furones, D., Roque, A., Pruzzo, C., Cheslett, D., Gdoura, R., Vallaeys, T., 2015. A European epidemiological survey of *Vibrio splendidus* clade shows unexplored diversity and massive exchange of virulence factors. World J. Microbiol. Biotechnol. http://dx.doi.org/10.1007/s11274-015-1800-y.

Nell J.A., 2002. Farming triploid oysters. Aquaculture, 210: 69-88.

Newcomb W.W., Juhas R.M., Thomsen D.R., Homa F.L., Burch A.D., Weller S.K., Brown J.C., 2001. The UL6 Gene Product Forms the Portal for Entry of DNA into the Herpes Simplex Virus Capsid. J. Virol., 75(22): 10923-10932.

Nicolas J.L., Comps M., Cochennec N., 1992. Herpes-like virus infecting Pacific oyster larvae, *Crassostrea gigas*. Bull. Eur. Ass. Fish Pathol. 12 (1), 11–13.

Noguerola I., Blanch A.R., 2008. Identification of Vibrio spp. with a set od dichotomous keys. J. Applied Microbiol., 105(1): 175-185. doi: 10/111/j.1365-2672.208.03730.

Normand J., 2005. Etude de la reproduction d'huîtres creuses triploïdes *Crassostra gigas* (Thunberg) : transmission des chromosomes et potentiel reproducteur. Rapport de stage de MII de l'Université de Bretagne Occidentale, 70p.

Normand J., Le Pennec M, Boudry P., 2008. Comparative histological study of gametogenesis in triploid oysters (*Crassostrea gigas*) reared in an estuarine farming site in France during the 2003 heatwave. Aquaculture, 282: 124-129. doi: 10.1016/j.aquaculture.2008.06.026.

Novoa B., Figueras A., 2000. Virus-like particles associated with mortalities of the carpet-shell clam *Ruditapes decussatus*. Dis. Aquat. Org., 39: 147-149.

Numachi K., Oizumi J., Sato S., Imai T., 1965. Studies on the Mass Mortality of the Oyster in Matsushima Bay III. The Pathological Changes of the Oyster Caused by Gram-Positive Bacteria and the Frequency of Their Infection. Bull. Tohoku Natl. Fish. Res. Inst. Shiogama 39–48.

O'Foighil D., Gaffney P.M., Wilbur A.E., Hilbish T.J., Pino M.A., 1998. Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea angulata*. Mar. Biol., 131: 497-503.

Oden E, Burioli EAV, Trancart S, Pitel PH, Houssin M., 2016. Multilocus sequence analysis of Vibrio splendidus related-strains isolated from blue mussel Mytilus sp. during mortality events. Aquaculture, 464, 420–427. doi: 10.1016/j.aquaculture.2016.07.024.

Oden E., Martenot C., Berthaux M., Travaillé E., Malas J.P., Houssin M., 2011. Quantification of ostreid herpesvirus (OsHV-1) in *Crassostrea gigas* by real-time PCR: Determination of a viral load threshold to prevent summer mortalities. Aquaculture, 317(1-4): 27-31. doi: 10.1016/j.aquaculture.2011.04.001.

OIE, Office International des Epizooties, 2014. Infection with Ostreid herpesvirus 1 microvariants (May 2014 version). Manual of Diagnostic for Aquatic Animals 2015. Chap.2.4.5. http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_ostreid_herpesvirus_1.htm.

Orr H.A., 1990. Why Polyploidy is Rarer in Animals Than in Plants. The American Naturalist, 136(6): 759-770.

Paillard C., Le Roux F., Borrego J.J., 2004. Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. Aquat. Living Resour., 17: 477-498. doi: 10.1051/alr:2004054.

Paludan S.R., Bowie A.G., Horan K.A., Recognition of herpesviruses by the innate immune system. Nat. Rev. Immunol., 11: 143-154.

Paul-Pont I., Dhand N., Whittington R.J., 2013b. Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture, 412: 202-214.

Paul-Pont I., Dhant N.K., Whittington R.J., 2013a. Spatial distribution of mortality in Pacific oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. Dis. Aquat. Orga., 105: 127-138.

Paul-Pont I., Evans O., Dhand N., Rubio A., Coad P., Whittington R., 2014. Descriptive epidemiology of mass mortality due to *Ostreid herpesvirus-1* (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas)* inbatista the Hawkesbury River estuary, Australia. Aquaculture, 422-423: 146-159. doi: 10.1016/j.aquaculture.2013.12.009.

Paul-Pont I., Evans O., Dhand N.K., Whittington R.J., 2015. Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian Ostreid herpesvirus-1 (OsHV-1) µVar strain. Dis. Aquat. Org., 113: 137-147. doi: 10.3354/dao02826.

Peeler E.J., Reese R.A., Cheslett D.L., Geoghegan F., Power A., Thrush M.A., 2012. Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 µVar in the Republic of Ireland in 2009. Prev. Vet. Med., 105(1-2): 136-143. doi: 10.1016/j.prevetmed.2012.02.001.

Pellett P.E., Roizman B., 2006. The Herpesviridae: a brief introduction. In: Knipe D.M., Howley P.M., Griffin D.E., Lamb R.A., Martin M.A., Roizman B., Strauss S.E. (Eds). Fields Virology, 5th edn. Lippincott, Williams & Wilkins, Philadelphia, pp 2479-2499.

Pépin J.F., Riou A., Renault T., 2008. Rapid and sensitive detection of Ostreid herpesvirus 1 in oyster samples by real-time PCR. J. Virol. Methods, 149: 269-276.

Pernet F., Barret J., Le Gall P., Corporeau C., Degremont L., Lagarde F., Pepin J.F., Keck N., 2012. Mass mortalities in Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau Lagoon, France. Aquac. Environ. Interact., 2: 215-237.

Pernet F., Barret J., Marty C., Moal J., Le Gall P., Boudry P., 2010. Environmental anomalies, energetic reserves and fatty acid modifications in oysters coincide with an exceptional mortality event. Mar. Ecol. Prog Ser., 401: 129-146. doi: 10.3354/meps08407.

Pernet F., Lupo C., Bacher C., Whittington R.J., 2016. Infectious diseases in oyster aquaculture require a new integrated approach. Phil. Trans. R. Soc. B, 371 : 20150213. doi: 10.1098/rstb.2015.0213.

Perng G.C., Jones C., Ciacci-Zanella J., Stone M., Henderson G., Yukht A., Slanina S.M., Hofman F.M., Ghiasi H., Nesburn A.B., Wechsler S.L., 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science, 287: 1500-1503. doi: 10.1126/science.287.5457.1500.

Petton B., Bernet F., Robert R., Boudry P., 2013. Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. Aquac. Environ. Interact., 3: 257-273. doi: 10.3354/aei00070.

Petton B., Boudry P., Alunno-Bruscia M., Pernet F., 2015a. Factors influencing disease-induced mortality of Pacific oysters *Crassostrea gigas*. Aquac. Environ. Interact., 6: 205-222.

Petton B., Bruto M., James A., Labreuche Y., Alunno-Bruscia M., Le Roux F., 2015b. *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. Front. Microbiol., 6: 686. doi: 10.3389/fmicb.2015.00686.

Philipp E.E.R., Kraemer L., Melzner F., Poustka A.J., Thieme S., Findeisen U., et al., 2012. Massively parallel RNA sequencing identifies a complex immune gene repertoire in the lophotrochozoan *Mytilus edulis*. PlosOne, 7 e33091.

Pichot Y., Comps M., Tige G., Grizel H., Rabouin M.A., 1980. Recherches sur B*onamia ostreae* Gen. N., sp. un., parasite nouveau de l'huitre plate *Ostrea edulis* L. Rev. Trav. Inst. Pêches Marit., 43 : 131-140.

Piffarer F., Beaumont A., Falguiere J.C., Flajshans M., Haffray P., Colombo L., 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture, 293(3-4): 125-156. doi: 10.1016/j.aquaculture.2009.04.036.

Piñeiro-Vidal M., Carballas C.G., Gómez-Barreiro O., Riaza A., Santos Y., 2008 *Tenacibaculum soleae* sp. nov., isolated from diseased sole (*Solea senegalensis* Kaup). Int. J. Syst. Evol. Microbiol., 58: 881–885.

Pitts J., 1994. Oyster industry cooperation keyed to FDA's cyto B approval. Fish Farming News 1(4), 2.

PROGIG, 2009. Prolifération de l'huître creuse du Pacifique *Crassostrea gigas* sur les côtes Manches-Atlantique françaises : bilan, dynamique, conséquences écologiques, économiques et ethnologiques, expériences et scénarios de gestion. Rapport de fin de contrat.
http://www1.liteau.net/uploads/projet_documents/LITEAU_II_2005_Hily_Synthese.pdf.

Rainbow P.S., Phillips D.J.H., 1993. Cosmopolitan biomonitors of trace metals. Mar. Pollut. Bull., 26: 593-601. doi: 10.1016/0025-326x(93)90497-8.

Ramsey J., Schemske D., 1998. Pathways, mechanisms and rates of polyploid formation in flowering plants. Annu. Rev. Ecol. Syst., 29: 467-501.

Randall R.E., Goodbourn S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol., 89(1): 1-47. doi: 10.1099/vir.0.83391-0.

Rasmussen L.P.D., 1986. Virus-associated granulocytomas in the marine mussel, *Mytilus edulis*, from three sites in Denmark. J. Invert. Pathol., 48: 117-123. Renault T., 1998. Infections herpétiques chez les invertébrés: détection de virus de types herpès chez les mollusques bivalves marins. Virologie, 2: 401-403.

Reece K.S., Cordes J.F., Stubbs J.B., Hudson K.L., Francis E.A., 2007. Molecular phylogenies help resolve taxonomic confusion with Asian *Crassostrea* oyster species. Mar. Biol., doi: 10.1007/s00227-007-0846-2.

Ren W., Chen C.H., Renault T., Cai Y., Bai C., Wang C., 2013. Complete genome sequence of Acute Viral Necrosis Virus associated with massive mortality outbreaks in the Chinese scallop *Chlamys farreri*. Virol. J. 10, 110.

Renault T., Allain G., Arzul I., Chollet B., Cobret L., De Decker S., et al., 2009. Summer mortality outbreaks of French Pacific Oyster, *Crassostrea gigas*, in 2008: research and detection of pathogens. Dis. Fish and Shellfish, 14th International Conference of the EAFP. Sept 14-19 Prague, Czeck Republic. abstract O: 112-127.

Renault T., Bouquet AL.L, Maurice J.T, Lupo C., Blachier P., 2014. Ostreid herpesvirus-1 infection among Pacific oyster (*Crassostrea gigas*) spat: Relevence of water temperature to virus replication and circulation prior to the onset of mortality. Appl. Environ. Microb., 80: 5419-5426.

Renault T., Faury N., Barbosa-Solomieu V., Moreau K., 2011. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, *Crassostrea gigas*, challenged with Ostreid herpesvirus 1. Dev. Comp. Immunol., 35: 725-735.

Renault T., Le Deuff R.M., Chollet B., Cochennec N., Gérard A., 2000. Concomitant herpes-like virus infection in hatchery-reared larvae and nursery-cultured spat *Crassostrea gigas*, *Ostrea edulis*. Dis. Aquat. Org., 42: 173-183.

Renault T., Le Deuff R.M., Cocgennec M., Chollet B., Maffart P., 1995. Herpes-like viruses associated with high mortality levels in larvae and spat of Pacific oyster, *Crassostrea gigas*: a comparative study, the thermal effects on virus detection in hatchery-reared larvae, reproduction of the disease in axenic larvae. Vet. Res., 26: 539-543.

Renault T., Le Deuff R.M., Lipart C., Delsert C., 2000. Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. J. Virol. Methods, 88: 41–50. doi:10.1016/S0166-0934(00)00175-0

Renault T., Lipart C., 1998. Diagnosis of herpes-like virus infections in oysters using molecular techniques. European Aquqculture Society, Special Publication, 26: 235-376.

Renault T., Lipart C., Arzul I., 2001. A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. Dis. Aquat. Org., 45: 1-7.

Renault T., Moreau P., Faury N., Pépin J.F., Segarra A., Webb S., 2012. Analysis of clinical Ostreid Herpevirus 1 (*Malacoherpesviridae*) specimens by sequencing amplified fragments from three virus genome areas. J. Virol., 86(10): 5942-5947. doi: 10.1128/JVI.06534-11.

Renault T., Novoa B., 2004. Viruses infecting bivalve molluscs. Aquat. Living Resour., 17: 397-409. doi: 10.1051/alr:2004049.

Renault, T., Cochennec, N., Le Deuff, R.M., Chollet, B., 1994a. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. Bull. Eur. Ass. Fish Pathol. 14(2), 64–66.

Renault, T., Le Deuff, R.M., Cochennec, N., Maffart, P., 1994b. Herpesviruses associated with mortalities among Pacific oyster, Crassostrea gigas, in France –comparative study. Rev. Med. Vet. 145 (10), 735–742.

REPAMO (REseau de PAthologie des MOllusques) (2013). Bilan 2012 du réseau Repamo. Ifremer. http://archimer.ifremer.fr/doc/00143/25470/23625.pdf.

REPAMO (REseau de PAthologie des MOllusques) (2014). Rapport d'activité 2015. http://wwz.ifremer.fr/sante_mollusques_eng/Documentation/Rapports-d-activite.

REPAMO (REseau de PAthologie des MOllusques) (2015). Rapport d'activité 2016. http://wwz.ifremer.fr/sante_mollusques_eng/Documentation/Rapports-d-activite.

Robalino J., Bartlett T., Shepard E., Prior S., Jaramillo G., Scura E., Chapman R.W., Gross P.S., Browdy C.L., Warr G.W., 2005. Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? J. Virol., 79(21):13561-71. doi: 10.1128/JVI.79.21.13561-13571.2005.

Roberts K.L., Baines J.D., 2011. Actin in herpesvirus infection. Viruses, 3: 336-346: doi: 10.3390/v3040336.

Robinson K.S., Clements A., Williams A.C., Berger C.N., Frankel G., 2011. Bax inhibitor 1 in apoptosis and disease. Oncogene, 30: 2391-2400.

Roizman B., Desrosiers R.C., Fleckenstein B., Lopez C., Minson A.C., Studdert M.J., 1992. Arch. Virol., 123(3-4): 425-449.

Roizman, B., Pellett, P.E., 2001. The family Herpesviridae: a brief introduction, In: Knipe, D.M., Howley, P.M. (Eds.), 4th ed. Fields Virology, vol. 2. Lippincott, Williams and Wilkins, Philadelphia, PA, pp. 2381–2398.

Roque A., Carrasco N., Andree K.B., Lacuesta B., Elandaloussi L., Gairin I., Rodgers C.J., Furones M.D., 2012. First report of OsHV-1 microvar in Pacific oyster (*Crassostrea gigas)* cultured in Spain. Aquaculture, 324-325: 303-306. doi: 10.1016/j.aquaculture.2011.10.018.

Rosani U., Varotto L., Domeneghetti S., Arcangeli G., Pallavicini A., Venier P., 2014. Dual analysis of host and pathogen transcriptomes in ostreid herpesvirus 1- positive *Crassostrea gigas*. Environ. Microb. doi: 10.1111/14622920.12706.

Saitou N., Nei M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4:406-425.

Sakoda A., Sakai Y., Hayakawa K., Suzuki M., 1997. Adsorption of viruses in water environment onto solid surfaces. Water Science and Technology, 35: 107-114.

Samain J.F., Dégremont L., Soletchnik P., Haure J., Bédier E., Ropert M., Moal J., Huvet A., Bacca H., Van Wormhoudt A., Delaporte M., Costil K., Pouvreau S., Lambert C., Boulo V., Soudant P., Nicolas J.L., Le Roux F., Renault T., Gagnaire B., Geret F., Boutet I., Burgeot T., Boudry P., 2007. Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture, 268: 227-243. doi:10.1016/j.aquaculture.2007.04.044.

Satheesh Kumar S., Ananda Bharathi R., Rajan J.J.S., Alavandi S.V., Poornima M., Balasubramanian C.P., Ponniah A.G., 2013. Viability of white spot syndrome virus (WSSV) in sediment during sun-drying (drainable pond) and under non-drainable pond conditions indicated by infectivity to shrimps. Aquaculture, 402-403: 119-126.

Saulnier D., De Decker S., Haffner P., 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: A useful tool for epidemiologic studies. J. Microbiol. Methods, 77: 191-197.

Sauvage C., Pépin J.F., Lapègue S., Boudry P., Renault T., 2009. Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: Differences in viral DNA detection and quantification using real-time PCR. Virus Res., 142: 181–187.

Savin K.W., Cocks B.G., Wong F., Sawbridge T., Cogan N., Savage D., Warner S., 2010. A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. Virol. J., 7: 308. doi:10.1186/1743-422X-7-308.

Schachtele S.J., Hu S., Little M.R., Lokensgard J.R., 2010. Herpes simplex virus induces neural oxidative damage via microlial cell Toll-like receptor-2. J. Neuroinflammation, 7: 35.

Schikorski D., Faury N., Pepin J.F., Saulnier D., Tourbiez D., Renault T., 2011. Experimental Ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas:* kinetics of virus DNA detection by qPCR in seawater and in oyster samples. Virus Research, 155: 28-34.

Schikorski D., Renault T., Saulnier D., Faury N., Moreau P., Pepin J.F., 2011. Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Vet. Res., 42: 27.

Schmitt P., Rosa R.D., Duperthuis M., de Lorgeril J., Bachère E., Destoumieux-Garzón D., 2012. The antimicrobial defense of the Pacific oyster, *Crassostrea gigas*. How diversity may compensate for scarcity in the regulation of Resident/Pathogenic microflora. Front. Microbiol., 3: 160-. doi: 10.3389/fmicb.2012.00160.

Segarra A., 2014a. Etude des interactions hôte/virus chez l'huitre creuse, *Crassostrea gigas*, et son virus Ostreid herpesvirus 1. Thèse.

Segarra A., Baillon L., Faury N., Tourbiez D., Renault T., 2016. Detection and distribution of ostreid herpesvirus 1 in experimentally infected Pacific oyster spat. J. Invertebr. Pathol., 133: 59-65.

Segarra A., Faury N., Pépin J.F., Renault T., 2014a. Transcriptomic study of 39 Ostreid herpesvirus 1 genes during an experimental infection. J. Invert. Pathol., 119: 5–11. doi: 10.1016/j.jip.2014.03.002.

Segarra A., Mauduit F., Faury N., Trancart S., Dégremont L., Tourbiez D., Haffner P., Barbosa-Solomieu, V., Pépin, J.-F., Travers, M.-A., Renault, T., 2014b. Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. BMC Genomics, 15: 580. doi:10.1186/1471-2164-15-580.

Segarra A., Pépin J.F., Arzul I., Morga B., Faury N., Renault T., 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res., 153: 92–99.

Seo J.K., Lee M.J., Go H.J., Do Kim G., Do Jeong H., Nam B.H., et al., 2013a. Purification and antimicrobial function of ubiquitin isolated from the gill of Pacific oyster, *Crassostrea gigas*. Mol. Immunol., 53: 88-98. doi: 10.1016/j.molimm.2012.07.003.

Seo J.K., Lee M.J., Nam B.H., Park N.G., 2013b. cgMolluscidin, a novel dibasic residue repeat rich antimicrobial peptide, purified from gill of the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol., 35: 480-488. doi: 10.1016/j.fsi.2013.05.010.

Severini A., Scraba D.G., Tyell D.L.J., 1996. Branched structures in the intracellular DNA of herpes simplex virus type 1. J. Virol., 70: 3169-3175.

Shieh M.T., WuDunn D., Montgomery R.I., Esko J.D., Spear P.G., 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell. Biol., 116(5): 1273-1381. PMC2289355.

Shimahara Y., Kurita J., Kiryu I., Nishioka T., Yuasa K., Kawana M., Kamaishi T., Oseko N.,2012. Surveillance of type 1 Ostreid Herpesvirus (OsHV-1) variants in Japan. Fish Pathol., 47(4): 129-136. doi: 10.3147/jsfp.47.129.

Shukla D., Liu J., Blaiklock P., Shworak N.W., Bai X., Esko J.D., Cohen G.H., Eisenberg R.J., Rosenberg R.D., Spear P.G., 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell, 99: 13–22. [PubMed: 10520990]

Shukla D., Spear P.G., 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J. Clin. Invest. 2001; 108:503–510. [PubMed: 11518721].

Smolowitz R., Leavitt D., Perkins F., 1998. Observations of a Protistan Disease Similar to QPX in *Mercenaria mercenaria* (Hard Clams) from the Coast of Massachusetts. J. Invertebr. Pathol. 71, 9–25. doi:10.1006/jipa.1997.4706.

Sokolava I.M., 2009. Apoptosis in molluscan immune defence. ISJ 6: 49-58.

Sokolova I.M., Evans E., Hughes F.M., 2004. Cadmium-induced apoptosis in oyster haemocytes involves distorbance of cellular energy balance but no mitochondrial permeability transition. J. Exp. Biol., 207: 3369-3380.

Soletchnik P., Huvet A., Le Moine O., Razet D., Geairon P., Faury N., Goulletquer P., Boudry P. ,2002. A comparative field study of growth, survival and reproduction of Crassostrea gigas, C. angulata and their hybrids. Aquat . Living Resour., 15: 243-250.

Soletchnik P., Ropert M., Mazurié J., Gildas Fleury P., Le Coz F., 2007. Relationships between oyster mortality patterns and environmental data from monitoring databases

along the coasts of France. Aquaculture, 271: 384-400. doi:10.1016/j.aquaculture.2007.02.049.

Spagnoli F., Specchiulli A., Scirocco T., Carapella G., Villani P., Casolino G., Schiavone P., Franchi M. (2002). The Lago di Varano: Hydrologic Characteristics and Sediment Composition. Marine Ecol., 23 (supl. 1): 384-394. doi: 10.1111/j.1439-0485.2002.tb00036.x.

Spear P.G., Eisenberg R. J., Cohen G.H., 2000. Three classes of cell surface receptors for alphaherpesvirus entry. Virology, 275:1-8.

Spear P.G., Longnecker R., 2003. Herpesvirus entry: an update. J. Virol., 77(19): 10179-10185. doi: 10.1128/JVI.77.19.10179-10185.2003.

Stanley J.G., Allen S.K., Hidu H., 1981. Polyploidy induced in the American oyster, *Crassostrea virginica*, with cytochalasin-B. Aquaculture, 23: 1-10.

Stasiak K., Demattei M.V., Federici B.A., Bigot Y., 2000. Phylogenetic position of the *Diadromus pulchellus* ascovirus DNA polymerase among viruses with large double-stranded DNA genomes. J. Gen. Virol., 81: 3059-3072. doi: 10.1099/0022-1317-81-12-3059.

Steinert S.A., 1996. Contribution of apoptosis to observed DNA damage in mussel cells. Mar. Environ. Res., 42(1-4): 253-259.

Stöck M., Lamatsch D.K., Steinlein C., Epplen J.T., Grosse W.R., Hock R., Klapperstück T., Lampert K.P., Scheer U., Schmid M., Schartl M., 2002. A bisexually reproducing all-triploid vertebrate. Nat. Genet., 30(3): 25-28. doi: 10.1038/ng839.

Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., Muroga, K., 1998. *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. Dis. Aquat. Org., 33 : 111–118. http://dx.doi.org/10.3354/dao033111.

Suquet M., de Kermoysan G., Gonzalez Araya R., Queau I., Lebrun L ., Le Souchu P., Mingant C., 2009. Anesthesia in Pacific oyster *Crassostrea gigas*. Aquat. Living Resour., 22 : 29-34.

Takahashi K., Mori K., Nomura T., 1986. Occurence and characterization of lysozyme from marine bivalves. Bull. Japanese Soc. Fisheries Sci., 52: 863-868.

Takahashi K.G., Nakamura A., Mori K., 2000. Inhibitory effects of ovoglubulins on bacillary necrosis in larvae of the Pacific oyster *Crassostrea gigas*. J. Invertebr. Pathol., 75: 212-217.

Tamura K., Nei M., and Kumar S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. P. Natl. Acad. Sci (USA), 101: 11030-11035.

Tan J., Lancaster M., Hyatt A., van Driel R., Wong F., Warner S., 2007. Purification of a herpes-like virus from abalone (*Haliotis* spp.) with ganglioneuritis and detection by transmission electron microscopy. J. Virol. Methods 2008, 149: 338-341. 10.1016/j.jviromet.2007.12.019.

Terahara K., Takahashi K.G., 2008. Mechanisms and immunological roles of apoptosis in molluscs. Curr. Pharm. Des. 14: 131-137.

Terahara K., Takahashi K.G., Mori K., 2005. Pacific oyster haemocytes undergo apoptosis following cell-adhesion mediated by integrin-like molecules. Comp. Biochem. Phys. A: 141(2): 215-222. doi: 10.1016/j.cbpb.2005.05.040.

Thiry E., Dubuisson J., Pastoret P.P., 1986. Pathogenesis, latency and reactivation of infections by herpesviruses. Rev. Sci. Tech. Off. Int. Epiz., 5(4): 809-819.

Thiry, E., Thiry, J., 2011. Herpesvirus infections in ruminants and elephants.

Thunberg C. P., 1793. Tekning och Beskrifning på en stor Ostronsort ifrån Japan. Kongliga Vetenskaps Academiens Nya Handlingar, 14(4-6): 140-142.

Tiscar P.G., Mosca F., 2004. Defence mechanisms in farmed marine molluscs. Vet. Res. Commun., 28: 57-62.

Tittel J.N., Steller H., 2000. A comparison of programmed cell death between species. Genome Biol., 1: 1-6.

Tiwari V., Clement C., Xu D., Valyi-Nagy T., Yue B.Y., Liu J., Shukla D., 2006. Role for 3-O-sulfated heparan sulfate as the receptor for herpes simplex virus type 1 entry into primary human corneal fibroblasts. J. Virol.; 80: 8970–8980. PubMed: 16940509.

Toranzo A.E., Magarinos B., Romalde J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture, 246: 37-61.

Travers M.A., Mersni Achour R., Haffner P., Tourbiez D., Cassone A.-L., Morga B., Doghri I., Garcia C., Renault T., Fruitier-Arnaudin I., Saulnier D., 2014. First description of French *V. tubiashii* strains pathogenic to mollusk: I. Characterization of isolates and detection during mortality events. J. Invertebr. Pathol., 123: 49-59. doi:10.1016/j.jip.2014.04.009

Tsurumi T., Fujita M., Kudoh A., 2004. Latent and lytic Epstein-Barr virus replication strategies. Rev. Med. Virol., 15: 3-15. doi: 10.1002/rmv.441

Vasquez-Yeomans R., Caceres-Martinez J., Figueras-Huerta A., 2004. Herpes-like virus associated with eroded gills of the Pacific oyster *Crassostrea gigas* in Mexico. J. Shellfish Res., 23: 417-419. doi: 10.3354/dao02189.

Venier P., Varotto L., Rosani U., Millino C., Celegato B., Bernante F., et al., 2011. Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. BMC Genomics, 12 : 69.

Vigerust D.J., Shepherd V.L., 2007. Virus glycosylation: role in virulence and immune interactions. Trends microbiol., 15: 211-218. doi: 10.1016/j.tim.2007.03.003.

Vigneron V., Solliec G., Montanié H., Renault T., 2004. Detection of Ostreid Herpesvirus 1 (OsHV-1) DNA in seawater by PCR: influence of water parameters in bioassays. Dis. Aquat. Org., 62: 35-44. doi: 10.3354/dao062035.

Wang C.M., Wang H.G., Song X.L., Wang Y.G. Huang J., Song W.B., 2002. Possible virus-like etiology causing massive death of scallop *Chlamys farreri* in North China. World Aquaculture 2002, Bejing China: 777.

Wang N., Li Y., Cai Y., Ren W., Wang C., 2010. Detection of acute viral necrosis virus (AVNV) in plankton using FQ-PCR during a molecular epidemiology survey. J. Fisheries of China, 34: 1566-1571.

Ward R.D., Zemlak T.S., Innes B.H., Last P.R., Hebert P.D.N, 2005. DNA barcoding Australia's fish species. Philos. Trans. R. Soc. B 360, 1847–1857.

Webb S.C., Filder A., Renault T., 2007. Primers forPCR-based detection of Ostreid herpes virus-1 (OsHV-1): Apllication in a survey of New Zealand molluscs. Aquaculture, 272: 126-139.

Weller S.K., Spadaro A., Schaffer J.E., Murray A.W., Maxam A.M., Schaffer P.A., 1985. Cloning, sequencing, and functional analysis of OriL, a herpes simplex virus type 1 origin of DNA replication. Mol. Cell. Biol., 5: 930-942. doi: 10.1128/MCB.5.5.930.

Weststrate M.W., Geelen J.L.M.C., van der Noordaa J., 1980. Human cytomegalovirus DNA: physical maps for restriction endonucleases *Bgl*II, *Hind*III and *Xba*I. J. Gen. Virol., 49: 1-21.

Whittington R.J., Hick P.M., Evans O., Rubio A., Alford B., Dhand N., Pal-Pont I., 2015. Protection of Pacific oyster (*Crassostrea gigas*) spat from mortality due to Ostreid herpesvirus 1 (OsHV-1 µVar) using simple treatments of incoming seawater in land-based upwellers. Aquaculture, 437: 10-20. doi: 10.1016/j.aquaculture.2014.11.016.

Winnepenninckx B., Backeljau T., De Wachter R., 1993. Extraction of high molecular weight DNA from molluscs. Trends Genet., 9(12): 407. doi: 10.1016/0168-9525(93)90102-N.

Wittmann G., 1986. Aujeszky's disease. Rev. Sci. Tech. Off. Int. Epiz., 5(4): 959-977.

Wu X., Xu X., Yu Z., Wei Z., Xia J., 2010. Comparison of seven *Crassostrea* mitogenomes and phylogenetic analyses. Mol. Phylogenet. Evol., 57: 448-454. doi: 10.106/j.ympev.2010.05.029.

Xia J.,2, Bai C., Wang C., Song X., Huang J., 2015. Complete genome sequence of Ostreid, 12:1 10. doi: 10.1186/s12985-015-0334-0.

Xu F., Wang Y., Feng Y., Huang W., Wang W., Li L., Fang X., Que H., Zhang G., 2014. Identification of conserved and novel MicroRNAs in the Pacific oyster *Crassostrea gigas* by deep sequencing. PlosOne 9, e104371. doi: 10.1371/journal.pone.0104371.

Yáñez R., Rodríguez J.M., Nogal M.L., Yuste L., Enríquez C., Rodríguez J.F., Viñuela E., 1995. Analysis of the complete nucleotide sequence of African swine fever virus. Virology, 208: 249-278. doi:10.1006/viro.1995.1149.

Yang S., Wu X., 2010. Identification and functional characterization of a human sTRAIL homolog, CasTRAIL, in an invertebrate oyster *Crassostrea ariakensis*. Dev. Comp. Immunol., 34: 538-545.

Yoshino T.P., Bickham U., Bayne C.J., 2013. Molluscan cells in culture: primary cell cultures and cell lines. Can. J. Zool., 91(6): 394-404. doi: 10.1139/cjz-2012-0258.

Yu Z., Kong X., Zhang L., Guo X., Xiang J., 2003. Taxonomic status of four *Crassostrea* oysters from China as inferred from mitochondrial sequences. J. Shellfish Res., 22(1): 31-38.

Zhan L., Li L., Zhu Y., Zhang G., Guo X., 2014. Transcriptome analysis reveals a rich gene set related to innate immunity in the Eastern oyster (*Crassostrea virginica*). Mar. Biotechnol. N.Y., 16: 17-33.

Zhang G., Fang X., Guo X., Li L., Luo R., Xu F., Yang P., Zhang L., et al., 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature, 490: 49–54. doi:10.1038/nature11413.

Zhang J., Dong S., Tian X., Dong Y., Liu X., Yan D., 2006. Studies on the rotifer (*Brachionus urceus* Linnaeus, 1758) as a vector in white spot syndrome virus (WSSV) transmission. Aquaculture, 261(4): 1181-1185. doi: 10.1016/j.aquaculture.2006.09.002.

Zhang J., Dong S., Tian X., Dong Y., Liu X., Yan D., 2007. Virus-phytoplankton adhesion: a new WSSV transmission route to zooplankton. Acta Oceanologica Sinica, 26(6): 109-115.

Zhang L., Li L., Guo X., Litman G.W., Dishaw L.J., Zhang G., 2015. Massive expansion and functional divergence of innate immune genes in a protostosome. Sci. Rep., 5: 8693. doi: 10.1038/srep08693.

Zhang L., Li L., Zhang G., 2011. Gene discovery, comparative analysis and expression profile reveal the complexity of the *Crassostrea gigas* apoptosis system. Dev. Comp. Immunol., 35 : 603-610.

Zhang L., Li L., Zhu Y., Zhang G., Guo X., 2013. Transcriptome analysis reveals rich gene set related to innate immunity in the eastern oyster (*Crassostrea virginica*). Mar. Biotechnol., 16: 17-33.

Zhou Z.H., He J., Jakana J., Tatman J.D., Rixon F.J., Chiu W., 1995. Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. Nat. Struct. Biol., 2(11): 1026-30.

APPENDIX A

A.1/ Mollusc samples collected from wild populations in Italy and analyses done

date	location	ref n°	number	species	type	G	Va	Os	Os seq
July 2012	Chioggia	/	60	*Crassostrea gigas*	W	x	x	x	x
July 2012	Cervia	/	60	*Crassostrea gigas*	W	x	x	x	x
July 2012	Fiorenzuola	/	60	*Crassostrea gigas*	W	x	x	x	x
July 2012	Capoiale-Varano	/	60	*Crassostrea gigas*	W	x	x	x	x
01 April 2014	Cavallino	33469	3	*Crassostrea gigas*	W	x			
01 April 2014	Lio Piccolo S.O.	33463	8	*Crassostrea gigas*	W	x			
01 April 2014	Lio Piccolo E	33460	14	*Crassostrea gigas*	W	x			
01 May 2014	Murano	44342	18	*Crassostrea gigas*	W				
01 May 2014	Chioggia	44343	1	*Crassostrea gigas*	W				
01 May 2014	Murano	44345	7	*Mytilus* sp.	W				
20 May 2014	Caorle	49351	14	*Crassostrea gigas*	W		x	x	x
20 May 2014	Caorle	49354	4	*Ostrea edulis*	W			x	
20 May 2014	Monfalcone	49356	30	*Crassostrea gigas*	W	x	x	x	x
20 May 2014	Chioggia	49359	30	*Crassostrea gigas*	W	x	x	x	x
20 May 2014	Muggia	49358	30	*Crassostrea gigas*	W	x	x	x	x
20 May 2014	P. Garibaldi	49361	30	*Crassostrea gigas*	W	x	x	x	x
03 June 2014	Caleri	55520	30	*Crassostrea gigas*	W	x	x	x	x
03 June 2014	Caleri	55518	5	*Crassostrea gigas*	W				
04 June 2014	Capoiale-Varano	55519	30	*Crassostrea gigas*	W	x	x	x	x
04 June 2014	Capoiale-Varano	55513	3	*Crassostrea gigas*	W				
09 June 2014	Caorle	56227	10	*Ostrea edulis*	W				
10 June 2014	P. S. Stefano	57179	30	*Ostrea edulis*	W				
19 June 2014	Cervia	59650	30	*Crassostrea gigas*	W		x	x	
19 June 2014	Cervia	59651	3	*Crassostrea gigas*	W				
26 June 2014	Orbetello	61525	30	*Crassostrea gigas*	W	x	x	x	
26 June 2014	Orbetello	61509	5	*Crassostrea gigas*	W				
03 July 2014	Marano	63233	30	*Crassostrea gigas*	W	x	x	x	x
23 July 2014	Giulianova	67895	30	*Crassostrea gigas*	W	x	x	x	x
11 June 2015	Caleri	53096	50	*Mytilus* sp.	W			x	
10 June 2015	Giulianova	53104	35	*Mytilus* sp.	W			x	
10 June 2015	Capoiale-Varano	53047	50	*Mytilus* sp.	W			x	
11 June 2015	Chioggia	53102	35	*Mytilus* sp.	W			x	
24 June 2015	La Spezia	56472	53	*Limaria tubercolata*	W			x	
24 June 2015	La Spezia	56457	29	*Patella* sp.	W			x	
24 June 2015	La Spezia	56436	20	*Chlamys varia*	W			x	
24 June 2015	La Spezia	56450	32	*Anomia ephippium*	W			x	
24 June 2015	La Spezia	56448	30	*Ostrea edulis*	W			x	
24 June 2015	La Spezia	56442	50	*Mytilus* sp.	W			x	
07 July 2015	Muggia	59151	1	*Ostrea edulis*	W			x	
10 July 2015	Marano	59163	50	*Mytilus* sp.	W			x	
11 July 2015	Chioggia	59166	50	*Ruditapes philippinarum*	W			x	
08 July 2015	Caorle	59134 A	8	*Chlamys glabra*	W			x	
08 July 2015	Caorle	59134 B	2	*Aequipecten opercularis*	W			x	
08 July 2015	Caorle	59168	18	*Anomia ephippium*	W			x	
08 July 2015	Caorle	59136	38	*Ostrea edulis*	W			x	
15 July 2015	San Teodoro	60901	50	*Ruditapes decussatus*	W			x	
15 July 2015	La Spezia	60947	32	*Ostrea edulis*	W			x	
21 July 2015	Caleri	62061	50	*Mytilus* sp.	W				
29 July 2015	La Spezia	63578	20	*Ostrea edulis*	W			x	
30 July 2015	Caleri	65015	50	*Ruditapes philippinarum*	W			x	
30 July 2015	Caleri	65014	11	*Parvicardium* sp.	W			x	
30 July 2015	Caleri	64014	6	*Solen marginatus*	W			x	

Analyses: G, genetic; H, histological; B, bacteriological; C, chemical; Va, PCR for V. *aestuarianus* ; Os, PCR for OsHV-1; Os seq, sequencing of 3 regions

G: genetic analysis for the oyster identification

Va: real-time PCR for the detection and quantification of *Vibrio aestuarianus* in flesh

Os: real-time PCR for the detection and quantification of OsHV-1 in flesh

Os seq: sequencing of three regions of the OsHV-1 genome

A.2/ Oyster samples from the 2014 monitoring campaign and analyses done

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
14 May 2014	Hatchery	49350.1	500	spat T5	triploid			x	x	
14 May 2014	Hatchery	49350.2	500	spat T5	diploid			x	x	
21 July 2014	Hatchery	66997.1	500	spat T5	triploid			x	x	
21 July 2014	Hatchery	66997.2	500	spat T5	diploid			x	x	
25 September 2014	Hatchery	82438	500	spat T5	triploid			x	x	
19 June 2014	Caorle	59616	30	spat	triploid			x	x	
19 June 2014	Caorle	59628	30	spat	diploid			x	x	
20 June 2014	Giulianova	59641	30	spat	triploid			x	x	
20 June 2014	Giulianova	59643	30	spat	diploid			x	x	
20 June 2014	Varano	59646	30	spat	triploid			x	x	x
20 June 2014	Varano	59647	30	spat	diploid			x	x	
21 June 2014	Caleri	59634	30	spat	triploid			x	x	
21 June 2014	Caleri	59638	30	spat	diploid			x	x	
26 June 2014	La Spezia	61529	30	spat	triploid			x	x	
26 June 2014	La Spezia	61526	30	spat	diploid			x	x	
26 June 2014	Orbetello	61523	30	spat	triploid			x	x	
26 June 2014	Orbetello	61521	30	spat	diploid			x	x	
27 June 2014	Gaeta	61519	30	spat	triploid			x	x	
27 June 2014	Gaeta	61516	30	spat	diploid			x	x	
04 July 2014	Giulianova	63219	30	spat	triploid			x	x	
04 July 2014	Giulianova	63226	30	spat	diploid			x	x	
04 July 2014	Varano	63203	30	spat	triploid			x	x	x
04 July 2014	Varano	63209	30	spat	diploid			x	x	
07 July 2014	Caleri	64049	30	spat	triploid			x	x	x
07 July 2014	Caleri	64047	30	spat	diploid			x	x	x
07 July 2014	Caleri	64045	3	spat	triploid	x	x	x	x	x
07 July 2014	Caorle	64035	30	spat	triploid			x	x	
07 July 2014	Caorle	64040	30	spat	diploid			x	x	
07 July 2014	San Teodoro	64483	30	spat	triploid			x	x	
07 July 2014	San Teodoro	64490	30	spat	diploid			x	x	
07 July 2014	Olbia	64507	30	spat	triploid			x	x	
07 July 2014	Olbia	64506	30	spat	diploid			x	x	
09 July 2014	La Spezia	64494	30	spat	triploid			x	x	x
09 July 2014	La Spezia	64492	30	spat	diploid			x	x	x
09 July 2014	Orbetello	64500	30	spat	triploid			x	x	
09 July 2014	Orbetello	64502	30	spat	diploid			x	x	
10 July 2014	Gaeta	65102	30	spat	triploid			x	x	
10 July 2014	Gaeta	65103	30	spat	diploid			x	x	
21 July 2014	Caleri	67218	3	spat	triploid	x	x	x	x	x
21 July 2014	Caleri	67227	30	spat	triploid			x	x	x
21 July 2014	Caleri	67221	30	spat	diploid			x	x	
21 July 2014	Caleri	67226	30	spat	triploid			x	x	
21 July 2014	Caleri	67219	30	spat	diploid			x	x	
21 July 2014	Caorle	67205	30	spat	triploid			x	x	
21 July 2014	Caorle	67212	30	spat	diploid			x	x	
21 July 2014	Caorle	67204	30	spat	triploid			x	x	
21 July 2014	Caorle	67201	30	spat	diploid			x	x	
23 July 2014	Varano	67908	30	spat	triploid			x	x	x
23 July 2014	Varano	67907	30	spat	diploid			x	x	
24 July 2014	Giulianova	67899	30	spat	triploid			x	x	
24 July 2014	Giulianova	67901	30	spat	diploid			x	x	
30 July 2014	La Spezia	69443	30	spat	triploid			x	x	
30 July 2014	La Spezia	69444	30	spat	diploid			x	x	
30 July 2014	La Spezia	69441	30	spat	triploid			x	x	
30 July 2014	La Spezia	69442	30	spat	diploid			x	x	x
30 July 2014	Orbetello	69436	30	spat	triploid			x	x	
30 July 2014	Orbetello	69437	30	spat	diploid			x	x	
30 July 2014	Orbetello	69433	30	spat	triploid			x	x	x
30 July 2014	Orbetello	69435	30	spat	diploid			x	x	
31 July 2014	Gaeta	69392	30	spat	triploid			x	x	
31 July 2014	Gaeta	69395	30	spat	diploid			x	x	
31 July 2014	Gaeta	69386	30	spat	triploid			x	x	
31 July 2014	Gaeta	69385	30	spat	diploid			x	x	

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
31 July 2014	Olbia	69430	30	spat	triploid			x	x	
31 July 2014	Olbia	69420	30	spat	diploid			x	x	
31 July 2014	Olbia	69419	30	spat	triploid			x	x	
31 July 2014	Olbia	69417	30	spat	diploid			x	x	
31 July 2014	San Teodoro	69412	30	spat	triploid			x	x	x
31 July 2014	San Teodoro	69410	30	spat	diploid			x	x	
31 July 2014	San Teodoro	69408	30	spat	triploid			x	x	
31 July 2014	San Teodoro	69407	30	spat	diploid			x	x	
06 August 2014	Caorle	70780	30	spat	diploid			x	x	
06 August 2014	Caorle	70776	30	spat	triploid			x	x	
06 August 2014	Caorle	70778	30	spat	diploid			x	x	
06 August 2014	Caleri	70765	30	spat	triploid			x	x	x
06 August 2014	Caleri	70766	30	spat	diploid			x	x	x
06 August 2014	Caleri	70767	30	spat	triploid			x	x	
06 August 2014	Caleri	70768	30	spat	diploid			x	x	
06 August 2014	Caorle	70777	30	spat	triploid			x	x	
07 August 2014	Varano	71455	30	spat	diploid			x	x	
07 August 2014	Varano	71458	30	spat	triploid			x	x	
07 August 2014	Varano	71459	30	spat	diploid			x	x	
07 August 2014	Varano	71460	30	spat	triploid			x	x	
07 August 2014	Giulianova	71463	30	spat	diploid			x	x	
07 August 2014	Giulianova	71465	30	spat	triploid			x	x	
07 August 2014	Giulianova	71478	30	spat	diploid			x	x	
07 August 2014	Giulianova	71479	30	spat	triploid			x	x	
28 August 2014	Gaeta	75070	30	spat	triploid			x	x	
28 August 2014	Gaeta	75066	30	spat	diploid			x	x	
28 August 2014	Gaeta	75065	30	spat	triploid			x	x	
28 August 2014	Gaeta	75064	30	spat	diploid			x	x	
28 August 2014	Orbetello	75091	30	spat	triploid			x	x	
28 August 2014	Orbetello	75086	30	spat	diploid			x	x	x
28 August 2014	Orbetello	75089	30	spat	triploid			x	x	
28 August 2014	Orbetello	75085	30	spat	diploid			x	x	
28 August 2014	La Spezia	75080	30	spat	triploid			x	x	x
28 August 2014	La Spezia	75076	30	spat	diploid			x	x	x
28 August 2014	La Spezia	75078	30	spat	triploid			x	x	
28 August 2014	La Spezia	75075	30	spat	diploid			x	x	x
02 September 2014	Caorle	76228	30	spat	diploid			x	x	
02 September 2014	Caorle	76227	30	spat	diploid			x	x	
02 September 2014	Caorle	76229	30	spat	triploid			x	x	
02 September 2014	Caorle	76230	30	spat	triploid			x	x	
02 September 2014	Caleri	76135	30	spat	triploid			x	x	x
02 September 2014	Caleri	76131	30	spat	diploid			x	x	x
02 September 2014	Caleri	76136	30	spat	triploid			x	x	x
02 September 2014	Caleri	76133	21	spat	diploid			x	x	
03 September 2014	Varano	76199	30	spat	triploid			x	x	
03 September 2014	Varano	76194	30	spat	diploid			x	x	
03 September 2014	Varano	76196	30	spat	triploid			x	x	
03 September 2014	Varano	76189	30	spat	diploid			x	x	
03 September 2014	San Teodoro	76220	30	spat	triploid			x	x	x
03 September 2014	San Teodoro	76215	30	spat	diploid			x	x	
03 September 2014	San Teodoro	76217	30	spat	triploid			x	x	
03 September 2014	San Teodoro	76212	30	spat	diploid			x	x	
03 September 2014	Olbia	76209	30	spat	triploid			x	x	x
03 September 2014	Olbia	76206	30	spat	diploid			x	x	
03 September 2014	Olbia	76207	30	spat	triploid			x	x	x
03 September 2014	Olbia	76202	30	spat	diploid			x	x	
17 September 2014	Giulianova	80604	30	spat	triploid			x	x	
17 September 2014	Giulianova	80603	30	spat	diploid			x	x	
17 September 2014	Giulianova	80601	30	spat	triploid			x	x	
17 September 2014	Giulianova	80599	30	spat	diploid			x	x	
23 September 2014	San Teodoro	82425	30	spat	triploid			x	x	x
23 September 2014	San Teodoro	82428	30	spat	diploid			x	x	x
23 September 2014	San Teodoro	82426	30	spat	triploid			x	x	x
23 September 2014	San Teodoro	82429	30	spat	diploid			x	x	
25 September 2014	La Spezia	82415	30	spat	triploid			x	x	x
25 September 2014	La Spezia	82417	30	spat	diploid			x	x	
25 September 2014	La Spezia	82413	30	spat	diploid			x	x	x
25 September 2014	La Spezia	82414	30	spat	triploid			x	x	
25 September 2014	Orbetello	82419	30	spat	triploid			x	x	x
25 September 2014	Orbetello	82422	30	spat	diploid			x	x	

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
25 September 2014	Orbetello	82420	30	spat	triploid			x	x	x
25 September 2014	Orbetello	82423	30	spat	diploid			x	x	x
25 September 2014	Gaeta	82654	30	spat	triploid			x	x	
25 September 2014	Gaeta	82658	30	spat	diploid			x	x	
25 September 2014	Gaeta	82660	30	spat	diploid			x	x	
25 September 2014	Gaeta	82661	30	spat	triploid			x	x	
30 September 2014	Caorle	84856	30	spat	diploid			x	x	
30 September 2014	Caorle	84857	30	spat	diploid	x	x	x	x	
30 September 2014	Caorle	84833	30	spat	triploid			x	x	
30 September 2014	Caorle	84877	30	spat	triploid			x	x	
30 September 2014	Caorle	84878	30	spat	triploid			x	x	
01 October 2014	Giulianova	84848	30	spat	triploid			x	x	
01 October 2014	Giulianova	84851	30	spat	diploid			x	x	
01 October 2014	Giulianova	84849	30	spat	triploid			x	x	
01 October 2014	Giulianova	84852	30	spat	diploid			x	x	
01 October 2014	Varano	84841	30	spat	triploid			x	x	x
01 October 2014	Varano	84845	30	spat	diploid	x	x	x	x	
01 October 2014	Varano	84842	30	spat	triploid			x	x	x
01 October 2014	Varano	84846	30	spat	diploid			x	x	x
01 October 2014	Olbia	84864	30	spat	triploid			x	x	
01 October 2014	Olbia	87900	30	spat	diploid			x	x	
01 October 2014	Olbia	87903	30	spat	triploid			x	x	
01 October 2014	Olbia	84866	30	spat	diploid			x	x	
01 October 2014	San Teodoro	84834	30	spat	triploid	x	x	x	x	
01 October 2014	San Teodoro	84838	30	spat	diploid			x	x	
01 October 2014	San Teodoro	84837	30	spat	triploid			x	x	
01 October 2014	San Teodoro	84839	30	spat	diploid			x	x	
02 October 2014	Caleri	84868	30	spat	triploid			x	x	x
02 October 2014	Caleri	84869	30	spat	diploid			x	x	
02 October 2014	Caleri	84870	30	spat	triploid			x	x	x
02 October 2014	Caleri	84872	30	spat	diploid			x	x	x
08 October 2014	Gaeta	86963	30	spat	triploid			x	x	
08 October 2014	Gaeta	86961	30	spat	diploid			x	x	
08 October 2014	Gaeta	86983	30	spat	triploid			x	x	
08 October 2014	Gaeta	86984	30	spat	diploid			x	x	
08 October 2014	Orbetello	86966	30	spat	triploid			x	x	
08 October 2014	Orbetello	86969	30	spat	diploid			x	x	
08 October 2014	Orbetello	86967	30	spat	triploid			x	x	
08 October 2014	Orbetello	86970	30	spat	diploid			x	x	
08 October 2014	La Spezia	86979	2	spat	triploid			x	x	
08 October 2014	La Spezia	86972	30	spat	triploid			x	x	
08 October 2014	La Spezia	86976	30	spat	diploid			x	x	
08 October 2014	La Spezia	86943	30	spat	triploid			x	x	
08 October 2014	La Spezia	86984	30	spat	diploid			x	x	
16 October 2014	Caleri	90150	30	spat	triploid			x	x	
16 October 2014	Caleri	90154	30	spat	diploid			x	x	
16 October 2014	Caleri	90155	30	spat	triploid			x	x	x
16 October 2014	Caleri	90156	6	spat	diploid			x	x	
16 October 2014	Caleri	90157	12	spat	triploid			x	x	
17 October 2014	Giulianova	90809	30	spat	triploid			x	x	
17 October 2014	Giulianova	90815	30	spat	diploid			x	x	
17 October 2014	Giulianova	90820	30	spat	triploid			x	x	
17 October 2014	Giulianova	90823	30	spat	diploid			x	x	
17 October 2014	Giulianova	90825	30	spat	triploid			x	x	
17 October 2014	Varano	90167	30	spat	triploid			x	x	
17 October 2014	Varano	90173	30	spat	diploid			x	x	
17 October 2014	Varano	90177	30	spat	triploid			x	x	
17 October 2014	Varano	90183	30	spat	diploid			x	x	
17 October 2014	Varano	90190	30	spat	triploid			x	x	
18 October 2014	Caorle	90504	30	spat	diploid			x	x	
18 October 2014	Caorle	90508	30	spat	diploid	x	x	x	x	
18 October 2014	Caorle	90513	30	spat	triploid			x	x	
18 October 2014	Caorle	90201	30	spat	triploid			x	x	
18 October 2014	Caorle	90202	30	spat	triploid			x	x	
23 October 2014	Gaeta	92621	30	spat	triploid			x	x	
23 October 2014	Gaeta	92619	30	spat	diploid			x	x	
23 October 2014	Gaeta	92701	30	spat	diploid			x	x	
23 October 2014	Gaeta	92703	30	spat	triploid			x	x	
23 October 2014	Gaeta	92715	30	spat	triploid			x	x	
23 October 2014	Orbetello	92624	30	spat	triploid			x	x	
23 October 2014	Orbetello	92628	30	spat	diploid			x	x	

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
23 October 2014	Orbetello	92625	30	spat	triploid			x	x	
23 October 2014	Orbetello	92630	30	spat	diploid			x	x	
23 October 2014	Orbetello	92623	30	spat	triploid			x	x	
23 October 2014	La Spezia	92632	30	spat	triploid			x	x	x
23 October 2014	La Spezia	92638	30	spat	diploid			x	x	x
23 October 2014	La Spezia	92649	30	spat	triploid			x	x	
23 October 2014	La Spezia	92650	30	spat	diploid			x	x	
23 October 2014	La Spezia	92651	30	spat	triploid			x	x	
23 October 2014	Olbia	92613	20	spat	triploid			x	x	
23 October 2014	Olbia	92607	30	spat	diploid			x	x	
23 October 2014	Olbia	92616	7	spat	triploid		x	x	x	
23 October 2014	Olbia	92614	30	spat	diploid			x	x	
23 October 2014	Olbia	92618	30	spat	triploid			x	x	
23 October 2014	San Teodoro	92603	30	spat	triploid			x	x	x
23 October 2014	San Teodoro	92601	30	spat	diploid			x	x	x
23 October 2014	San Teodoro	92597	30	spat	triploid			x	x	x
23 October 2014	San Teodoro	92594	30	spat	diploid			x	x	x
23 October 2014	San Teodoro	92599	30	spat	triploid			x	x	x

G: genetic analysis for the oyster identification

Va: real-time PCR for the detection and quantification of *Vibrio aestuarianus* in flesh

Os: real-time PCR for the detection and quantification of OsHV-1 in flesh

Os seq: sequencing of three regions of the OsHV-1 genome

H: histological examination

B: bacteriological analysis

A.3/ Oyster samples from the 2015 monitoring campaign and analyses done

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
14 April 2015	Hatchery	36221.1	500	spat T5	triploid			x	x	
14 April 2015	Hatchery	36221.2	500	spat T2	diploid			x	x	
14 September 2015	Hatchery	74176	500	spat T5	triploid			x	x	
21 January 2015	San Teodoro	sal1	18	>12 months	triploid			x	x	x
11 March 2015	San Teodoro	sal2	12	>12 months	triploid			x	x	x
20 March 2015	San Teodoro	sal3	3	>12 months	triploid			x	x	x
27 March 2015	San Teodoro	sal4	15	>12 months	triploid			x	x	x
31 March 2015	San Teodoro	sal5	7	>12 months	triploid			x	x	
03 April 2015	San Teodoro	sal6	6	>12 months	triploid			x	x	
07April 2015	San Teodoro	32012	30	>12 months	triploid			x	x	
07April 2016	San Teodoro	32014	30	>12 months	diploid			x	x	
14 April 2015	Caorle	36232	30	>12 months	triploid			x	x	
14 April 2015	Caorle	36231	30	>12 months	diploid			x	x	
14 April 2015	Caleri	36226	30	>12 months	triploid			x	x	
14 April 2015	Caleri	36227	30	>12 months	diploid			x	x	
14 April 2015	Gaeta	36225	30	>12 months	triploid			x	x	
14 April 2015	Gaeta	36248	30	>12 months	diploid			x	x	
16 April 2015	Orbetello	36182	30	>12 months	triploid			x	x	
16 April 2015	Orbetello	36183	30	>12 months	diploid			x	x	
16 April 2015	La Spezia	36189	30	>12 months	triploid			x	x	
16 April 2015	La Spezia	36188	30	>12 months	diploid			x	x	
18 May 2015	Caorle	46196	30	>12 months	triploid			x	x	
18 May 2015	Caorle	46195	30	>12 months	diploid			x	x	
18 May 2015	Caorle	46198	30	spat	triploid			x	x	
18 May 2015	Caorle	46194	30	spat	diploid			x	x	
18 May 2015	Caleri	46208	30	>12 months	triploid			x	x	x
18 May 2015	Caleri	46207	30	>12 months	diploid			x	x	x
18 May 2015	Caleri	46210	30	spat	triploid			x	x	x
18 May 2015	Caleri	46213	30	spat	diploid			x	x	x
25 May 2015	San Teodoro	48946	30	>12 months	triploid			x	x	x
25 May 2015	San Teodoro	48947	30	>12 months	diploid			x	x	x
25 May 2015	San Teodoro	48948	30	spat	triploid			x	x	x
25 May 2015	San Teodoro	48950	30	spat	diploid			x	x	x
26 May 2015	Orbetello	48954	30	>12 months	triploid			x	x	x
26 May 2015	Orbetello	48955	30	>12 months	diploid			x	x	x
26 May 2015	Orbetello	48953	30	spat	triploid			x	x	x
26 May 2015	Orbetello	48951	30	spat	diploid			x	x	x
26 May 2015	La Spezia	48944	30	>12 months	triploid			x	x	
26 May 2015	La Spezia	48943	30	>12 months	diploid			x	x	
26 May 2015	La Spezia	48940	30	spat	triploid			x	x	
26 May 2015	La Spezia	48941	30	spat	diploid			x	x	
03 June 2015	Meuvaines	EBMV-063N	30	spat	triploid			x	x	
03 June 2015	Meuvaines	EBMV-062N	30	spat	diploid			x	x	
09 June 2015	Caorle	53080	30	>12 months	triploid			x	x	
09 June 2015	Caorle	53439	30	>12 months	diploid			x	x	
09 June 2015	Caorle	53079	30	spat	triploid			x	x	
09 June 2015	Caorle	53083	30	spat	diploid			x	x	
10 June 2015	Giulianova	53108	30	spat	triploid			x	x	
10 June 2015	Giulianova	53106	30	spat	diploid			x	x	
10 June 2015	Varano	53077	30	spat	triploid			x	x	
10 June 2015	Varano	53073	30	spat	diploid			x	x	
11 June 2015	Baie des Veys	EBBD-063N	30	spat	triploid			x	x	
11 June 2015	Baie des Veys	EBBD-062N	30	spat	diploid			x	x	
11 June 2015	Caleri	53087	30	spat	triploid			x	x	x
11 June 2015	Caleri	53091	30	spat	diploid			x	x	x
15 June 2015	San Teodoro	54301	30	>12 months	triploid			x	x	x
15 June 2015	San Teodoro	54298	30	>12 months	diploid			x	x	x
15 June 2015	San Teodoro	54303	30	spat	triploid			x	x	x
15 June 2015	San Teodoro	54305	30	spat	diploid			x	x	x
24 June 2015	Orbetello	56426	30	>12 months	triploid			x	x	x
24 June 2015	Orbetello	56421	30	>12 months	diploid			x	x	x
24 June 2015	Orbetello	56422	30	spat	triploid			x	x	x
24 June 2015	Orbetello	56420	30	spat	diploid			x	x	x
24 June 2015	La Spezia	56466	30	>12 months	triploid			x	x	
24 June 2015	La Spezia	56465	30	>12 months	diploid			x	x	
24 June 2015	La Spezia	56458	30	spat	triploid			x	x	
24 June 2015	La Spezia	56464	30	spat	diploid			x	x	

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
25 June 2015	Gaeta	56418	30	>12 months	triploid			x	x	
25 June 2015	Gaeta	56414	30	>12 months	diploid			x	x	
25 June 2015	Gaeta	56409	30	spat	triploid			x	x	
25 June 2015	Gaeta	56143	30	spat	diploid			x	x	
01 July 2015	Meuvaines	EBMV-073N	30	spat	triploid			x	x	x
01 July 2015	Meuvaines	EBMV-072N	30	spat	diploid			x	x	x
02 July 2015	Baie des Veys	EBBD-073N	30	spat	triploid			x	x	x
02 July 2015	Baie des Veys	EBBD-072N	30	spat	diploid			x	x	x
08 July 2015	Caorle	59143	30	>12 months	triploid	x	x	x	x	
08 July 2015	Caorle	59138	30	>12 months	diploid	x	x	x	x	
08 July 2015	Caorle	59140	30	spat	triploid			x	x	
08 July 2015	Caorle	59142	30	spat	diploid			x	x	
08 July 2015	Caleri	59145	30	spat	triploid	x	x	x	x	x
08 July 2015	Caleri	59144	30	spat	diploid	x	x	x	x	x
15 July 2015	Orbetello	60935	30	>12 months	triploid			x	x	x
15 July 2015	Orbetello	60934	30	>12 months	diploid			x	x	x
15 July 2015	Orbetello	60938	30	spat	triploid			x	x	x
15 July 2015	Orbetello	60940	30	spat	diploid			x	x	x
15 July 2015	La Spezia	60951	30	>12 months	triploid	x	x	x	x	
15 July 2015	La Spezia	60948	30	>12 months	diploid	x	x	x	x	x
15 July 2015	La Spezia	60952	30	spat	triploid			x	x	x
15 July 2015	La Spezia	60954	30	spat	diploid			x	x	x
15 July 2015	San Teodoro	60898	30	>12 months	triploid	x	x	x	x	x
15 July 2015	San Teodoro	60897	30	>12 months	diploid	x	x	x	x	x
15 July 2015	San Teodoro	60815	30	spat	triploid	x	x	x	x	x
15 July 2015	San Teodoro	60896	30	spat	diploid	x	x	x	x	x
29 July 2015	Orbetello	63562	30	>12 months	triploid			x	x	
29 July 2015	Orbetello	63563	30	>12 months	diploid			x	x	
29 July 2015	Orbetello	63569	30	spat	triploid			x	x	
29 July 2015	Orbetello	63566	30	spat	diploid			x	x	
29 July 2015	La Spezia	63575	30	>12 months	triploid			x	x	
29 July 2015	La Spezia	63574	30	>12 months	diploid			x	x	
29 July 2015	La Spezia	63579	30	spat	triploid			x	x	
29 July 2015	La Spezia	63573	30	spat	diploid			x	x	
30 July 2015	Caorle	65007	30	>12 months	triploid			x	x	
30 July 2015	Caorle	65006	30	>12 months	diploid			x	x	
30 July 2015	Caorle	64021	30	spat	triploid			x	x	
30 July 2015	Caorle	64020	30	spat	diploid			x	x	
30 July 2015	Caleri	64018	30	spat	triploid			x	x	
30 July 2015	Caleri	64017	30	spat	diploid			x	x	
03 August 2015	Meuvaines	EBMV-083N	30	spat	triploid			x	x	x
03 August 2015	Meuvaines	EBMV-082N	30	spat	diploid			x	x	x
03 August 2015	Baie des Veys	EBBD-083N	30	spat	triploid			x	x	x
03 August 2015	Baie des Veys	EBBD-082N	30	spat	diploid			x	x	
05 August 2015	San Teodoro	65013	30	>12 months	triploid			x	x	
05 August 2015	San Teodoro	65011	30	>12 months	diploid			x	x	x
05 August 2015	San Teodoro	65010	30	spat	triploid			x	x	
05 August 2015	San Teodoro	65009	30	spat	diploid			x	x	
12 August 2015	Orbetello	66188	30	>12 months	triploid			x	x	
12 August 2015	Orbetello	66189	30	>12 months	diploid			x	x	
12 August 2015	Orbetello	66191	30	spat	triploid			x	x	x
12 August 2015	Orbetello	66190	30	spat	diploid			x	x	
12 August 2015	La Spezia	66194	30	>12 months	triploid			x	x	
12 August 2015	La Spezia	66193	30	>12 months	diploid			x	x	
12 August 2015	La Spezia	66196	30	spat	triploid	x	x	x	x	
12 August 2015	La Spezia	66195	30	spat	diploid			x	x	
17 August 2015	Caorle	67213	30	>12 months	triploid			x	x	
17 August 2015	Caorle	67212	30	>12 months	diploid			x	x	
17 August 2015	Caorle	67214	30	spat	triploid			x	x	
17 August 2015	Caorle	67216	30	spat	diploid			x	x	
17 August 2015	Caleri	67220	30	spat	triploid			x	x	x
17 August 2015	Caleri	67221	30	spat	diploid			x	x	x
03 September 2015	San Teodoro	70742	30	>12 months	triploid	x	x	x	x	x
03 September 2015	San Teodoro	70743	30	>12 months	diploid	x	x	x	x	
03 September 2015	San Teodoro	70747	30	spat	triploid	x	x	x	x	x
03 September 2015	San Teodoro	70746	30	spat	diploid	x	x	x	x	x
03 September 2015	La Spezia	70603	30	>12 months	triploid	x	x	x	x	
03 September 2015	La Spezia	70607	30	>12 months	diploid	x	x	x	x	
03 September 2015	La Spezia	70601	30	spat	triploid	x	x	x	x	
03 September 2015	La Spezia	70602	30	spat	diploid	x	x	x	x	

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
03 September 2015	Orbetello	70600	30	>12 months	triploid			x	x	
03 September 2015	Orbetello	70596	30	>12 months	diploid			x	x	
03 September 2015	Orbetello	70599	30	spat	triploid			x	x	
03 September 2015	Orbetello	70597	30	spat	diploid			x	x	x
04 September 2015	Caorle	70859	30	>12 months	triploid	x	x	x	x	
04 September 2015	Caorle	70857	30	>12 months	diploid	x	x	x	x	
04 September 2015	Caorle	70858	30	spat	triploid	x	x	x	x	
04 September 2015	Caorle	70856	30	spat	diploid	x	x	x	x	
04 September 2015	Caleri	70864	30	spat	triploid	x	x	x	x	x
04 September 2015	Caleri	70862	30	spat	diploid	x	x	x	x	x
15 September 2015	Meuvaines	EBMV-093N	30	spat	triploid			x	x	x
15 September 2015	Meuvaines	EBMV-092N	30	spat	diploid			x	x	x
16 September 2015	Baie des Veys	EBBD-093N	30	spat	triploid			x	x	x
16 September 2015	Baie des Veys	EBBD-092N	30	spat	diploid			x	x	x
16 September 2015	San Teodoro	73909	30	>12 months	triploid			x	x	x
16 September 2015	San Teodoro	73907	30	>12 months	diploid			x	x	x
16 September 2015	San Teodoro	73903	30	spat	triploid			x	x	x
16 September 2015	San Teodoro	73905	30	spat	diploid			x	x	x
16 September 2015	Orbetello	73901	30	>12 months	triploid			x	x	x
16 September 2015	Orbetello	73902	30	>12 months	diploid			x	x	x
16 September 2015	Orbetello	73898	30	spat	triploid			x	x	
16 September 2015	Orbetello	73896	30	spat	diploid			x	x	x
16 September 2015	La Spezia	73881	30	>12 months	triploid			x	x	x
16 September 2015	La Spezia	73885	30	>12 months	diploid			x	x	x
16 September 2015	La Spezia	73889	30	spat	triploid			x	x	x
16 September 2015	La Spezia	73887	30	spat	diploid			x	x	
16 September 2015	Caorle	73922	30	>12 months	triploid			x	x	
16 September 2015	Caorle	73923	30	>12 months	diploid			x	x	
16 September 2015	Caorle	73937	30	spat	triploid			x	x	
16 September 2015	Caorle	73921	30	spat	diploid			x	x	
16 September 2015	Caleri	73948	30	spat	triploid			x	x	x
16 September 2015	Caleri	73941	30	spat	diploid			x	x	x
28 September 2015	San Teodoro	77590	30	>12 months	triploid			x	x	
28 September 2015	San Teodoro	77585	30	>12 months	diploid			x	x	x
28 September 2015	San Teodoro	77610	30	spat	triploid			x	x	x
28 September 2015	San Teodoro	77604	30	spat	diploid			x	x	x
06 October 2015	Caleri	82195	30	spat	triploid			x	x	x
06 October 2015	Caleri	82194	30	spat	diploid			x	x	x
06 October 2015	Caleri	82196	30	spat sept	triploid			x	x	
07 October 2015	Caorle	82200	30	>12 months	triploid			x	x	
07 October 2015	Caorle	82199	30	>12 months	diploid			x	x	
07 October 2015	Caorle	82202	30	spat	triploid			x	x	
07 October 2015	Caorle	82204	30	spat	diploid			x	x	
14 October 2015	Orbetello	83334	30	>12 months	triploid			x	x	
14 October 2015	Orbetello	83335	30	>12 months	diploid			x	x	
14 October 2015	Orbetello	83328	30	spat	triploid			x	x	x
14 October 2015	Orbetello	83330	30	spat	diploid			x	x	x
14 October 2015	Orbetello	83333	30	spat sept	triploid			x	x	
15 October 2015	La Spezia	83326	27	>12 months	triploid	x	x	x	x	x
15 October 2015	La Spezia	83327	25	>12 months	diploid	x	x	x	x	x
15 October 2015	La Spezia	83318	2	spat	triploid	x	x	x	x	x
15 October 2015	La Spezia	83321	5	spat	diploid	x	x	x	x	x
15 October 2015	La Spezia	83325	30	spat sept	triploid	x	x	x	x	
26 October 2015	San Teodoro	87798	30	>12 months	triploid	x	x	x	x	x
26 October 2015	San Teodoro	87796	30	>12 months	diploid	x	x	x	x	x
26 October 2015	San Teodoro	87789	30	spat	triploid	x	x	x	x	x
26 October 2015	San Teodoro	87793	30	spat	diploid	x	x	x	x	x
26 October 2015	San Teodoro	87801	30	spat sept	triploid			x	x	
29 October 2015	Giulianova	88814	30	spat	triploid			x	x	
29 October 2015	Giulianova	88797	30	spat	diploid			x	x	
29 October 2015	Varano	88799	30	spat	triploid			x	x	
29 October 2015	Varano	88798	30	spat	diploid			x	x	
02 November 2015	San Teodoro	90689	20	>12 months	triploid		x	x	x	
02 November 2015	San Teodoro	90692	30	>12 months	diploid	x	x	x	x	
18 November 2015	San Teodoro	97993	30	>12 months	triploid			x	x	
18 November 2015	San Teodoro	97995	30	>12 months	diploid			x	x	
18 November 2015	San Teodoro	98001	30	spat	triploid			x	x	
18 November 2015	San Teodoro	98004	30	spat	diploid			x	x	
18 November 2015	San Teodoro	97998	30	spat sept	triploid			x	x	
18 November 2015	Caorle	95908	30	>12 months	triploid	x	x	x	x	

date	location	ref n°	number	age/size	type	H	B	Va	Os	Os seq
18 November 2015	Caorle	95907	30	>12 months	diploid			x	x	
18 November 2015	Caorle	95909	30	spat	triploid			x	x	
18 November 2015	Caorle	95910	30	spat	diploid			x	x	
18 November 2015	Caorle	95904	30	spat sept	triploid			x	x	
18 November 2015	Caleri	95911	30	spat	triploid			x	x	
18 November 2015	Caleri	95913	30	spat	diploid			x	x	
18 November 2015	Caleri	95914	30	spat sept	triploid			x	x	
04 December 2015	La Spezia	101559	30	>12 months	triploid			x	x	
04 December 2015	La Spezia	101561	30	>12 months	diploid			x	x	
04 December 2015	La Spezia	101555	30	spat sept	triploid			x	x	
05 December 2015	Orbetello	101554	30	>12 months	triploid			x	x	
05 December 2015	Orbetello	101544	30	>12 months	diploid			x	x	
05 December 2015	Orbetello	101549	30	spat	triploid			x	x	
05 December 2015	Orbetello	101547	30	spat	diploid			x	x	
05 December 2015	Orbetello	101545	30	spat sept	triploid			x	x	
05 December 2015	Gaeta	101543	30	>12 months	triploid			x	x	
05 December 2015	Gaeta	101541	30	>12 months	diploid			x	x	
05 December 2015	Gaeta	101542	30	spat	triploid			x	x	
05 December 2015	Gaeta	101526	30	spat	diploid			x	x	
16 December 2015	San Teodoro	sal7	13	>12 months	triploid			x	x	x
16 December 2015	San Teodoro	sal8	5	spat	triploid			x	x	
16 January 2016	San Teodoro	sal9	24	>12 months	triploid			x	x	
16 January 2016	San Teodoro	sal10	12	spat	triploid			x	x	

G: genetic analysis for the oyster identification

Va: real-time PCR for the detection and quantification of *Vibrio aestuarianus* in flesh

Os: real-time PCR for the detection and quantification of OsHV-1 in flesh

Os seq: sequencing of three regions of the OsHV-1 genome

H: histological examination

B: bacteriological analysis

APPENDIX B

B.1/ Mollusc tissue processing and DNA extraction

Mollusc individuals are shucked and pools of large portion of tissue from the mantle, gills, heart, and adductor muscle are minced using scalpels (Burioli *et al*., 2016). If DNA extraction is not performed immediately, homogenates are frozen at -80°C until analysis.
For each sample, a mass of 50 mg of minced tissue is subjected to DNA extraction using a QIAamp DNA minikit® (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol for blood or body fluids, except for elution performed in 60 µL Qiagen elution buffer AE (Martenot *et al*., 2010).

An extraction control was systematically included during each extraction to prevent false-positives and consisted of 50 µL of DNA/nuclease-free water treated as a sample.

Extraction workflow:

- Weigh 50±0.5 mg of minced tissue in a 1.5 mL microtube
- Add 20 µL of proteinase K provided in the kit
- Add 200 µL of buffer AL provided in the kit
- Mix thoroughly by vortexing
- Incubate at 56°C for 10 min. At the end of the incubation time, tissues should be completely lysed
- Make a short-spin centrifugation to eliminate aerosol
- Add 200 µL of ethanol 99%
- Mix by vortexing for at least 10 sec
- Make a short-spin centrifugation to eliminate aerosol
- Transfer the mixture with a pipet from the microtube to a spin column provided in the kit
- Centrifuge at 6000 x g for 1 min
- Discard the flow-through and the collection tube and replace the spin column on a new collection tube provided in the kit
- Add 500 µL of buffer AW1 provided in the kit
- Centrifuge at 6000 x g for 1 min
- Discard the flow-through and the collection tube and replace the spin column on a new collection tube provided in the kit
- Add 500 µL of buffer AW2 provided in the kit
- Centrifuge at 20,000 x g for 3 min

- Discard the flow-through and the collection tube and replace the spin column on a new collection tube provided in the kit
- Centrifuge at 20,000 x g for 1 min
- Discard the flow-through and the collection tube and replace the column on a new 1.5 mL microtube
- Add 60 µL of elution buffer AE, provided in the kit, in the centre of the column and incubate for 5 min
- Centrifuge at 6000 x g for 1 min

DNA extracts are stored at -80°C.

B.2/ Quantitative real-time PCR for OsHV-1

This PCR assay permits the quantification of OsHV-1 DNA directly in mollusc tissues with a quantitation limit of 18 Genomic Units (GU)/mg of tissue and a detection limit of 6 GU/mg of tissues. However, it does not allow the discrimination between the variants.

The real-time PCR protocol is based on TaqMan® technology (Applied Biosystems) (Martenot *et al*., 2010) that targets the B region encoding a putative apoptosis inhibitor (Arzul *et al*., 2001).

Primer pair:

- B3 5’ GTCGCATCTTTGGATTTAACAA 3’
- B4 5’ ACTGGGATCCGACTGACAAC 3’

Probe:

- B3-B4 5’ TGCCCCTGTCATCTTGAGGTATAGACAATC 3’

Internal control probe:

- IC 5’ ATCGGGGGGGGGGGTTTTTTTTTTATCG 3’

Reaction volume (25 µL):

- 12.5 µL of Takara Premix Ex TaqTM 2X (Takara Bio Inc., Shiga, Japan)
- 0.5 µL of each primer (20 µM)
- 0.5 µL of B3-B4 probe (10 µM)
- 0.5 µL of IC probe (10 µM)
- 6.5 µL of DNA/nuclease-free water
- 2 µL of an IC solution (1.4 x 10^2 genome units (GU)/2 µL) consisting of a synthesized sequence containing the complementary sequence of the forward and reverse primers at each end and internally the IC probe sequence
- 2 lL of extracted DNA

Thermal cycling conditions:

- 1 cycle at 95 °C for 10 s
- 40 cycles at 95 °C for 5 s and at 60 °C for 20 s

A negative control is included in each assay and consisted of 2 µL of DNA/nuclease-free water in 23 µL of real-time PCR mix. The extraction control is treated as a sample.

To obtain the standard curve, different dilutions of plasmidic DNA solution, corresponding to the OsHV-1 target region, are used from 10 to 10^5 copies of DNA units/µL.

The run is validated only if the extraction and the negative controls did not present any amplification.

A sample is considered positive only if the difference between the Ct value of the duplicates did not exceed 0.5, the regression coefficient of the standard curve is at least 0.98 and the slope between -4.115 and -2.839, and the Ct value is ≤38.5. A sample is considered a true negative only if amplification of the IC was successful.

After referring to the standard curve, the viral load is expressed in genome units GU/50 mg of oyster tissue.

B.3/ Genomic exploration for the characterisation of OsHV-1 variants

The characterisation of the OsHV-1 microvariants is based on two target regions, the C region (including ORFs 4/5) and ORFs 42/43 (OIE, 2014). A third region of the virus genome, showing the deletion of two ORFs, has been commonly investigated (Renault *et al*., 2012).

Conventional PCRs targeting these three regions are performed and followed by amplicon sequencing.

Primer pairs:

- C2 (forward) 5' CTCTTTACCATGAAGATACCCACC 3' Arzul (2001)
- C6 (reverse) 5' GTGCACGGATTACCATTTTT 3'

- IA1 (forward) 5' CGCGGTTCATATCCAAAGTT 3' Segarra *et al*. (2010)
- IA2 (reverse) 5' AATCCCCATGTTTCTTGCTG 3'

- Del35.38-F 5' ATACGATGCGTCGGTAGAGC 3' Renault *et al*. (2012)
- Del35.38-R 5' TTACAGGAATGGGGTTCTCG 3'

Reaction volume (50 µL):

- 25 µL of 2x QIAGEN® Multiplex PCR Pre Mix (Qiagen)
- 1 µL of each primer (20 µM)
- 19 µL of DNA/nuclease-free water
- 4 µL of extracted DNA eluate

PCR conditions:

- activation/initial denaturation at 95 °C for 15 min;
- 40 amplification cycles at 95 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s
- final extension at 72 °C for 3 min

A negative control consisting of 4 µL of DNA/nuclease-free water in 46 µL of PCR mix is included.

Amplification is performed using T100^{TM} Thermo Cycler (Bio-Rad).

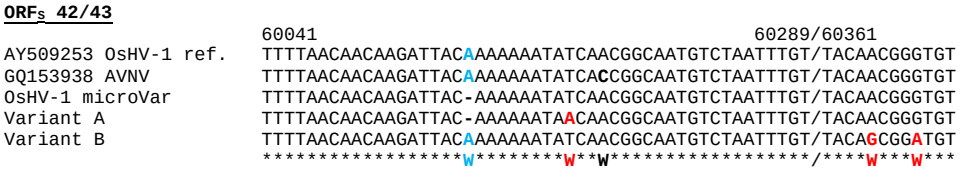
The evaluation of amplification success and correct fragment length is performed througth QIAxcel® system analysis (Qiagen).

Amplicons of expected length are sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing both strands. DNA sequencing accuracy is measured by the Phred quality score (Q score) and only scores above 30 are considered. The consensus sequence is determined by the alignment with ClustalW 1.81 (http://www.genome.jp/tools/clustalw/) and the comparison of both strand sequences.

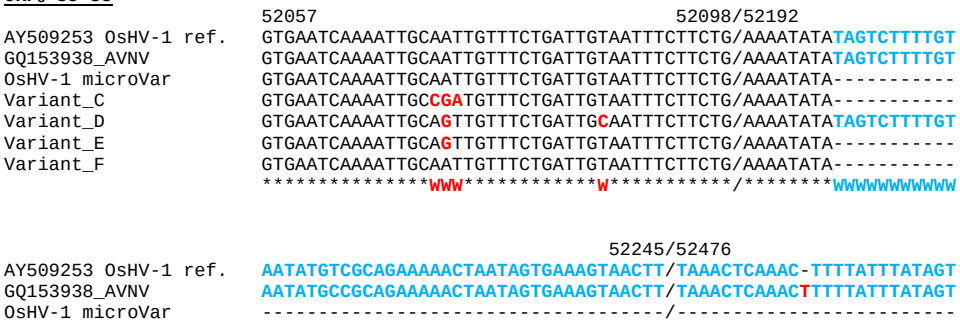
APPENDIX C

C.1/ Sequence alignment of the 3 regions (C, ORFs 42/43, and 35-38) in wild oysters

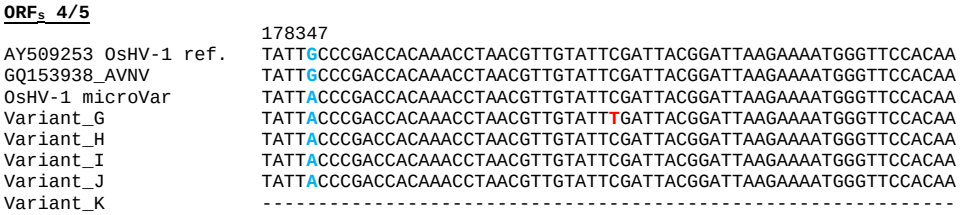
ORFs 42/43



ORFs 35-38



ORFs 4/5



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Variant_L               ------------------------------------------------------------
                        WWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWW

AY509253 OsHV-1 ref.    TCTAAAATTAAAAAAACCACATGGGGGCCAAGGAATTTAAA-CCCCGGGG-AAAAAGTATAA
GQ153938_AVNV           TCTAAAATT-AAAAAACCACATGGAGGCCAAGGAATTTAAA--CCCCGGG-AAAAAGTATAA
OsHV-1 microVar         TCTAAAATT-AAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAA
Variant_G               TCTAAAATT-AAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCCGGGAAAAAAGTATAA
Variant_H               TCTAAAATT-AAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAA
Variant_I               TCTAAAATT-AAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAA
Variant_J               TCTAAAATT-AAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAA
Variant_K               ------------------------------------------------------------
Variant_L               ------------------------------------------------------------
                        WWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWWW

AY509253 OsHV-1 ref.    ATAGGCGCGATTTGTCAGTTTAGAATCATACCCACACACTCAATCTCGAGTATACCACAACT
GQ153938_AVNV           ATAGGCGCGATTTGTCAGTTTAGAATCATACCCACACACTCAATCTCGAGTATACCACAACT
OsHV-1 microVar         ATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACAACT
Variant_G               ATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACAACT
Variant_H               ATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACAACT
Variant_I               ATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACAACT
Variant_J               ATAGGCGCGATTTGTCAGTTTAAAATCATACCCACAC--TCAATCTCGAGTATACCACAACT
Variant_K               -------------GTCAGTTTAGAATCATACCCACACACTCAATCTCGAGTATACCACAACT
Variant_L               -------------GTCAGTTTAGAATCATACCCACACACTCAATCTCGAGTATACCACAACT
                        WWWWWWWWWWWWW*********W**************WW**********************

AY509253 OsHV-1 ref.    GCTAAATTAACAGCATCTACTACTACTACTACTACTACTACTGAAAAAATGCAGCCTTTCAC
GQ153938_AVNV           GCTAAATTAACAGCATCTACTACTACT---------------G-AAAAATGCAGCCTTTCAC
OsHV-1 microVar         GCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCTTTCAC
Variant_G               GCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCTTTCAC
Variant_H               GCTAAATTAACAGCATCTACTACTACTACTACT---------G-AAAAATGCAGCCTTTCAC
Variant_I               GCTAAATTAACAGCATCTACTACTACT---------------G-AAAAATGCAGCCTTTCAC
Variant_J               GCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCTTTCAC
Variant_K               GCTAAATTAACAGCATCTACTACTACTACTACTACT------GAAAAAATGCAGCCTTTCAC
Variant_L               GCTAAATTAACAGCATCTACTACTACT---------------GAAAAAATGCAGCCTTTCAC
                        **************************WWWWWWWWWWWWWWW*W******************

AY509253 OsHV-1 ref.    AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
GQ153938_AVNV           AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
OsHV-1 microVar         AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
Variant_G               AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
Variant_H               AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
Variant_I               AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
Variant_J               AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
Variant_K               AGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATCAACCAGA
Variant_L               AGAATTTTGCGCCTTGATCAAAGCCATCACATCAGCCAGTAACGACTTTTTCATCAACCAGA
                        **********W******W********************W**********************

                                                                              178716
AY509253 OsHV-1 ref.    CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCGATTGCGAAGATAAAGTC
GQ153938_AVNV           CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCGATTGCGAAGATAAAGTC
OsHV-1 microVar         CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTC
Variant_G               CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTC
Variant_H               CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTC
Variant_I               CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTC
Variant_J               CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTC
Variant_K               CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCGATTGCGAAGATAAAGTC
Variant_L               CGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCGATTGCGAAGATAAAGTC
                        ****************************************W*****W***********
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C.2/ Sequence alignment of the OsHV-1 C Region, multisite tests, year 2014

*isolated during a mortality event

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Caleri 1*       TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
Caleri 2*       TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
Caleri 3        TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
Caleri 4*       TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
Caleri 5*       TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
San Teodoro1*   TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
San Teodoro2*   TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
La Spezia       TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
Caleri6         TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
Caleri7*        TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
µVar            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
AY509253        TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATA
                ************************************************************

Caleri1*        ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
Caleri2*        ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
Caleri3         ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
Caleri4*        ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
Caleri5*        ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
San Teodoro1*   ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
San Teodoro2*   ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
La Spezia       ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
Caleri6         ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
Caleri7*        ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
µVar            ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
AY509253        ATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTAT
                ************************************************************

Caleri1*        TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
Caleri2*        TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
Caleri3         TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
Caleri4*        TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
Caleri5*        TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
San Teodoro1*   TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
San Teodoro2*   TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
La Spezia       TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
Caleri6         TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
Caleri7*        TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
µVar            TACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
AY509253        TGCCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCCACAAT
                *W************************************************************

Caleri1*        CTAAAATTAAAAACCCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
Caleri2*        CTAAAATTAAAAACCCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
Caleri3         CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
Caleri4*        CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
Caleri5*        CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
San Teodoro1*   CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
San Teodoro2*   CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
La Spezia       CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
Caleri6         CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
Caleri7*        CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
µVar            CTAAAATTAAAAA-CCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATA
AY509253        CTAAAATTAAAAAAACCACATGGGGGCCAAGGAATTTAAA-CCCC-GGGGAAAAAGTATA
                *************WW************************W****W***W**********

Caleri1*        AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
Caleri2*        AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
Caleri3         AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
Caleri4*        AATAGGCGCGATTTGTCAGTTTAAAATCATACCCACAC--TCAATCTCGAGTATACCACA
Caleri5*        AATAGGCGCGATTTGTCAGTTTAAAATCATACCCACAC--TCAATCTCGAGTATACCACA
San Teodoro1*   AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
San Teodoro2*   AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
La Spezia       AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
Caleri6         AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
Caleri7*        AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
µVar            AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCACA
AY509253        AATAGGCGCGATTTGTCAGTTTAGAATCATACCCACACACTCAATCTCGAGTATACCACA
                **********************W**************WW********************

Caleri1*        ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
Caleri2*        ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
Caleri3         ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
Caleri4*        ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
Caleri5*        ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
San Teodoro1*   ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
San Teodoro2*   ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
La Spezia       ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
Caleri6         ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
Caleri7*        ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
µVar            ACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AAAAATGCAGCCT
AY509253        ACTGCTAAATTAACAGCATCTACTACTACTACTACTACTACTACTGAAAAAATGCAGCCT
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                 **********************************WWWWWWWWWWWW*W**********

Caleri1*         TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
Caleri2*         TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
Caleri3          TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
Caleri4*         TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
Caleri5*         TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
San Teodoro1*    TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
San Teodoro2*    TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
La Spezia        TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
Caleri6          TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
Caleri7*         TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
µVar             TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
AY509253         TTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCAGCCAGCAACGACTTTTTCATC
                 ************************************************************

Caleri1*         AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
Caleri2*         AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
Caleri3          AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
Caleri4*         AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
Caleri5*         AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
San Teodoro1*    AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
San Teodoro2*    AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
La Spezia        AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
Caleri6          AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
Caleri7*         AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
µVar             AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCAATTGCAAAGAT
AY509253         AACCAGACGAGGTTAACATGCGACATTTGTAAAGAGCTCGTCTCTTTCGATTGCGAAGAT
                 ************************************************W*****W*****

Caleri1*         AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
Caleri2*         AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
Caleri3          AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
Caleri4*         AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
Caleri5*         AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
San Teodoro1*    AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
San Teodoro2*    AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
La Spezia        AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTTACGGAACGC
Caleri6          AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
Caleri7*         AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
µVar             AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
AY509253         AAAGTCGTGGCATCATTGGCTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGC
                 ************************************************W*********

Caleri1*         AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
Caleri2*         AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
Caleri3          AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
Caleri4*         AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
Caleri5*         AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
San Teodoro1*    AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
San Teodoro2*    AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
La Spezia        AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
Caleri6          AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
Caleri7*         AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
µVar             AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
AY509253         AAAGACCTGAACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCACT
                 ************************************************************

Caleri1*         CTCATTGACGAAT
Caleri2*         CTCATTGACGAAT
Caleri3          CTCATTGACGAAT
Caleri4*         CTCATTGACGAAT
Caleri5*         CTCATTGACGAAT
San Teodoro1*    CTCATTGACGAAT
San Teodoro2*    CTCATTGACGAAT
La Spezia        CTCATTGACGAAT
Caleri6          CTCATTGACGAAT
Caleri7*         CTCATTGACGAAT
µVar             CTCATTGACGAAT
AY509253         CTCATTGACGAAT
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C.3/ Sequence alignment of the OsHV-1 ORFs 42/43, multisite tests, year 2014

*isolated during a mortality event

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Caleri1*          TGGTTTATATTTTTTGTAA-GCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri2*          TGGTTTATATTTTTTGTAA-GCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri3           TGGTTTATATTTTTTGTAA-GCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
La Spezia         TGGTTTATATTTTTTGTAA-GCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri4*          TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri5*          TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro1*     TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro2*     TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
µVar              TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
AY509253          TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
                  ******************W****************************************

Caleri1*          AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri2*          AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri3           AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
La Spezia         AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri4*          AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri5*          AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro1*     AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro2*     AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
µVar              AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
AY509253          AGATTACAAAAAAATATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
                  ************W***********************************************

Caleri1*          TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri2*          TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri3           TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
La Speezia        TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri4*          TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri5*          TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro1*     TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro2*     TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
µVar              TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
AY509253          TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
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Caleri1*          CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri2*          CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri3           CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
La Spezia         CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri4*          CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri5*          CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro1*     CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro2*     CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
µVar              CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
AY509253          CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
                  ************************************************************

Caleri1*          CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri2*          CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri3           CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
La Spezia         CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri4*          CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri5*          CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro1*     CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro2*     CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
µVar              CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
AY509253          CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
                  ************************************************************

Caleri1*          GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri2*          GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri3           GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
La Spezia         GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri4*          GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri5*          GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro1*     GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro2*     GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
µVar              GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
AY509253          GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
                  ************************************************************

Caleri1*          TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri2*          TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri3           TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
92632             TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri4*          TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri5*          TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro1*     TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro2*     TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
µVar              TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
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AY509253          TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
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Caleri1*          GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri2*          GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri3           GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
La Spezia         GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri4*          GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri5*          GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro1*     GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro2*     GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
µVar              GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
AY509253          GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
                  ************************************************************

Caleri1*          CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri2*          CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri3           CTTTGCTTCAAAATACGACAATAGCGATCTAT
La Spezia         CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri4*          CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri5*          CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro1*     CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro2*     CTTTGCTTCAAAATACGACAATAGCGATCTAT
µVar              CTTTGCTTCAAAATACGACAATAGCGATCTAT
AY509253          CTTCGCTTCAAAATACGACAATAGCGATCTAT
                  ***W**************************
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C.4/ Sequence alignment of the OsHV-1 ORFs 35-38, multisite tests, year 2014

*isolated during a mortality event

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Caleri3          ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
Caleri2*         ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
La Spezia        ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
Caleri4*         ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
San Teodoro2*    ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
Caleri1*         ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
AY509253         ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
µVar             ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCCT
                 ************************************************************


Caleri3          TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
Caleri2*         TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
La Spezia        TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
Caleri4*         TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
San Teodoro2*    TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
Caleri1*         TGAGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
AY509253         TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
µVar             TGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCTG
                 **W*********************************************************


Caleri3          TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
Caleri2*         TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
La Spezia        TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
Caleri4*         TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
San Teodoro2*    TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
Caleri1*         TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
AY509253         TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
µVar             TAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCCA
                 ************************************************************

                                                                      52215/
Caleri3          TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
Caleri2*         TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
La Spezia        TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
Caleri4*         TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
San Teodoro2*    TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
Caleri1*         TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
AY509253         TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATAGTCTTTTGTAATATG/
µVar             TGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA---------------/
                 ************************************************************

                 52755
Caleri3          ---------------------------------------------CTATCTTTTTGGCATT
Caleri2*         ---------------------------------------------CTATCTTTTTGGCATT
La Spezia        ---------------------------------------------CTATCTTTTTGGCATT
Caleri4*         ---------------------------------------------CTATCTTTTTGGCATT
San Teodoro2*    ---------------------------------------------CTATCTTTTTGGCATT
Caleri1*         ---------------------------------------------CTATCTTTTTGGCATT
AY509253         TAATACTTCGGGGCTGAACGGTGGTACATTGGTTACATCTTTTACTATCTTTTTGGCATT
µVar             ---------------------------------------------CTATCTTTTTGGCATT
                 ************************************************************

Caleri3          GATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
Caleri2*         GATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
La Spezia        GATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
Caleri4*         GATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
San Teodoro2*    GATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
Caleri1*         GATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
AY509253         GATGATTATGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
µVar             GATGATTATGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATCTTCCCATAA
                 ********W***************************************************

Caleri3          TGGATATTCCGTGTTTACAG
Caleri2*         TGGATATTCCGTGTTTACAG
La Spezia        TGGATATTCCGTGTTTACAG
Caleri4*         TGGATATTCCGTGTTTACAG
San Teodoro2*    TGGATATTCCGTGTTTACAG
Caleri1*         TGGATATTCCGTGTTTACAG
AY509253         TGGATATTCCATGTTTACAG
µVar             TGGATATTCCATGTTTACAG
                 **********W*********
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C.5/ Sequence alignment of the OsHV-1 C Region, multisite tests, year 2015

*isolated during a mortality event

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San Teodoro1            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro2            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro3            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
Caleri1                 TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro4            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro5*           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro6            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
Caleri2                 TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro7            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro8            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
Caleri3                 TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
Caleri4*                TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro9            TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro10           TTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAA
Caleri5*                TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro11*          TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
Caleri6                 TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro12*          TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro13           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro14*          TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San teodoro15           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San teodoro16           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San teodoro17           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
La Spezia*              TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San teodoro18           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
Caleri7                 TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro19           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
San Teodoro20           ??????????????????????????????????????????????????
AY509253                TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
µVar HQ842610           TTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAA
                        **************************************************

San Teodoro1            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro2            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro3            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri1                 AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro4            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro5*           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro6            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri2                 AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro7            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro8            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri3                 AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri4*                AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro9            AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro10           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri5                 AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro11*          AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri6                 AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro12*          AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro13           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro14*          AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro15           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro16           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro17           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
La Spezia               AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro18           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
Caleri7                 AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro19           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
San Teodoro20           ??????????????????????????????????????????????????
AY509253                AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
µVar HQ842610           AGTTGGGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATG
                        **************************************************

San Teodoro1            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro2            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro3            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri1                 GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro4            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro5*           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro6            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri2                 GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro7            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro8            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri3                 GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri4*                GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro9            GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro10           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri5                 GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro11*          GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri6                 GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
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San Teodoro12*          GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro13           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro14*          GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro15           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro16           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro17           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
La Spezia               GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro18           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
Caleri7                 GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro19           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
San Teodoro20           ??????????????????????????????????????????????????
AY509253                GCAAGATACACAATGAGCTATTGCCCGACCACAAACCTAACGTTGTATTC
µVar HQ842610           GCAAGATACACAATGAGCTATTACCCGACCACAAACCTAACGTTGTATTC
                        **********************x**************************

San Teodoro1            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro2            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro3            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri1                 GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro4            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro5*           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro6            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri2                 GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro7            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro8            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri3                 GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri4*                GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro9            GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro10           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri5                 GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro11*          GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri6                 GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro12*          GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro13           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro14*          GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro15           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro16           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro17           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Le Spezia               GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro18           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
Caleri7                 GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro19           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
San Teodoro20           ???????????AAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
AY509253                GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAAAACCAC
µVar HQ842610           GATTACGGATTAAGAAAATGGGTTCCACAATCTAAAATTAAAAA-CCCAC
                        *******************************************xx****

San Teodoro1            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro2            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro3            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri1                 ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro4            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro5*           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro6            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri2                 ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro7            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro8            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri3                 ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri4*                ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro9            ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro10           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri5                 ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro11*          ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri6                 ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro12*          ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro13           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro14*          ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro15           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro16           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro17           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
La Spezia               ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro18           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
Caleri7                 ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro19           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
San Teodoro20           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
AY509253                ATGGGGGCCAAGGAATTTAAA-CCCC-GGGGAAAAAAGTATAAATAGGCGC
µVar HQ842610           ATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAGTATAAATAGGCGC
                        **********************x*****x***x******************

San Teodoro1            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro2            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro3            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
Caleri1                 GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro4            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro5*           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro6            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
Caleri2                 GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro7            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro8            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
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Caleri3                 GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
Caleri4*                GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro9            GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro10           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
Caleri5                 GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro11*          GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
Caleri6                 GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro12*          GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro13           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro14*          GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro15           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro16           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro17           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
La Spezia               GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro18           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
Caleri7                 GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro19           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
San Teodoro20           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
AY509253                GATTTGTCAGTTTAGAATCATACCCACACACTCAATCTCGAGTATACCAC
µVar HQ842610           GATTTGTCAGTTTAGAATCATACCCACAC--TCAATCTCGAGTATACCAC
                        ****************************XX******************

San Teodoro1            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro2            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro3            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri1                 AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro4            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro5*           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro6            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri2                 AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro7            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro8            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri3                 AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri4*                AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro9            AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro10           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri5                 AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro11*          AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri6                 AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro12*          AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro13           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro14*          AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro15           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro16           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro17           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
La Spezia               AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro18           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
Caleri7                 AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro19           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
San Teodoro20           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
AY509253                AACTGCTAAATTAACAGCATCTACTACTACTACTACTACTACTGAAA
µVar HQ842610           AACTGCTAAATTAACAGCATCTACTACTACTACT------------G-AA
                        **********************************XXXXXXXXXXXX*X**

San Teodoro1            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro2            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro3            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri1                 AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro4            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro5*           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro6            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri2                 AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro7            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro8            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri3                 AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri4*                AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro9            AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro10           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri5                 AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro11*          AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri6                 AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro12*          AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro13           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro14*          AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro15           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro16           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro17           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
La Spezia               AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro18           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
Caleri7                 AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro19           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
San Teodoro20           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
AY509253                AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
µVar HQ842610           AAATGCAGCCTTTCACAGAATTTTGCACCTTGACCAAAGCCATCACATCA
                        **************************************************

San Teodoro1            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro2            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro3            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
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Caleri1                 GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro4            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro5*           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro6            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
Caleri2                 GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro7            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro8            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
Caleri3                 GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
Caleri4*                GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro9            GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro10           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
Caleri5                 GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro11*          GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
Caleri6                 GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro12*          GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro13           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro14*          GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro15           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro16           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro17           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
La Spezia               GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro18           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
Caleri7                 GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro19           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
San Teodoro20           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
AY509253                GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
µVar HQ842610           GCCAGCAACGACTTTTTCATCAACCAGACGAGGTTAACATGCGACATTTG
                        **************************************************

San Teodoro1            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro2            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro3            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri1                 TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro4            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro5*           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro6            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri2                 TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro7            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro8            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri3                 TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri4*                TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro9            TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro10           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri5                 TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro11*          TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri6                 TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro12*          TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro13           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro14*          TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro15           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro16           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro17           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
La Spezia               TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro18           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
Caleri7                 TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro19           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
San Teodoro20           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
AY509253                TAAAGAGCTCGTCTCTTTCGATTGCGAAGATAAAGTCGTGGCATCATTGG
µVar HQ842610           TAAAGAGCTCGTCTCTTTCAATTGCAAAGATAAAGTCGTGGCATCATTGG
                        ******************X*****X************************

San Teodoro1            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro2            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro3            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri1                 CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro4            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro5*           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro6            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri2                 CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro7            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro8            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri3                 CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri4*                CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro9            CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro10           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri5                 CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro11*          CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri6                 CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro12*          CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro13           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro14           CTGCAGTCAGATCTGACATACCCATAGAAGTTACGGAACGCAAAGACCTG
San Teodoro15           CTGCAGTTAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro16           CTGCAGTTAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro17           CTGCAGTTAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
La Spezia               CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro18           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
Caleri7                 CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro19           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
San Teodoro20           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
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AY509253                CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
µVar HQ842610           CTGCAGTCAGATCTGACATACCCATAGAAGTCACGGAACGCAAAGACCTG
                        *******x**********************x******************

San Teodoro1            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro2            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro3            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri1                 AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro4            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro5*           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro6            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri2                 AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro7            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro8            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri3                 AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri4*                AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro9            AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro10           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri5                 AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro11*          AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri6                 AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro12*          AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro13           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro14*          AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro15           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro16           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro17           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
La Spezia               AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro18           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
Caleri7                 AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro19           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
San Teodoro20           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
AY509253                AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
µVar HQ842610           AACCTCCTCGACCTGATCCAGTTCTTCGAAAAGAAGATAGAGTTTACCAC
                        **************************************************

San Teodoro1            TCTCATTGACGAAT
San Teodoro2            TCTCATTGACGAAT
San Teodoro3            TCTCATTGACGAAT
Caleri1                 TCTCATTGACGAAT
San Teodoro4            TCTCATTGACGAAT
San Teodoro5*           TCTCATTGACGAAT
San Teodoro6            TCTCATTGACGAAT
Caleri2                 TCTCATTGACGAAT
San Teodoro7            TCTCATTGACGAAT
San Teodoro8            TCTCATTGACGAAT
Caleri3                 TCTCATTGACGAAT
Caleri4*                TCTCATTGACGAAT
San Teodoro9            TCTCATTGACGAAT
San Teodoro10           TCTCATTGACGAAT
Caleri5                 TCTCATTGACGAAT
San Teodoro11*          TCTCATTGACGAAT
Caleri6                 TCTCATTGACGAAT
San Teodoro12*          TCTCATTGACGAAT
San Teodoro13           TCTCATTGACGAAT
San Teodoro14*          TCTCATTGACGAAT
San Teodoro15           TCTCATTGACGAAT
San Teodoro16           TCTCATTGACGAAT
San Teodoro17           TCTCATTGACGAAT
La Spezia               TCTCATTGACGAAT
San Teodoro18           TCTCATTGACGAAT
Caleri7                 TCTCATTGACGAAT
San Teodoro19           TCTCATTGACGAAT
San Teodoro20           TCTCATTGACGAAT
AY509253                TCTCATTGACGAAT
µVar HQ842610           TCTCATTGACGAAT
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C.6/ Sequence alignment of the OsHV-1 ORFs 42/43, multisite tests, year 2015

*isolated during a mortality event

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Caleri3             TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri4*            TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro12*      TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro8        TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri7             TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro10       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro14*      TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro17       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro5*       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro11*      TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro4        TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro15       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro1        TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro16       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro21       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro19       TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri1             TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri6             TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
Caleri5             TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
San Teodoro7        TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
La Spezia2          TGGTTTATATTTTTTGTAAAGCTTTTATATATCGTCAAATCCGGAAGTGTTTTAACAACA
AY509253            TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
µVar                TGGTTTATATTTTTTGTAAAGCTTTTATATATCTTCAAATCCGGAAGTGTTTTAACAACA
                    **********************************X**************************

Caleri3             AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri4*            AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro12*      AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro8        AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri7             AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro10       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro14*      AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro17       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro5*       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro11*      AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro4        AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro15       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro1        AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro16       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro21       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro19       AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri1             AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri6             AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
Caleri5             AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
San Teodoro7        AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
La Spezia2          AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
AY509253            AGATTACAAAAAAATATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
µVar                AGATTACAAAAAA-TATCAACGGCAATGTCTAATTTGTTCATTCCCCGATCTACCAAACG
                    ************X*********************************************

Caleri3             TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri4*            TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro12*      TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro8        TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri7             TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro10       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro14*      TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro17       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro5*       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro11*      TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro4        TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro15       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro1        TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro16       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro21       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro19       TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri1             TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri6             TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
Caleri5             TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
San Teodoro7        TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
La Spezia2          TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
AY509253            TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
µVar                TGCAGTCTACGACGGCCCTTTGCCAATGGTAGGCTCTTCCCTGCCGCCAATAGAAATAAA
                    ************************************************************

Caleri3             CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri4*            CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro12*      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro8        CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri7             CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro10       CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
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San Teodoro14*     CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro17      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro5*      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro11*     CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro4       CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro15      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro1       CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro16      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro21      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro19      CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri1            CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri6            CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
Caleri5            CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
San Teodoro7       CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
La Spezia2         CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATATTAA
AY509253           CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
µVar               CAGCAAAGGTGATAAATCGGTAGTTTATCTCAGGGGTGATGATCAACCAATTGATGTTAA
                   ************************************************X****

Caleri3            CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri4*           CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro12*     CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro8       CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri7            CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro10      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro14*     CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro17      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro5*      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro11*     CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro4       CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro15      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro1       CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro16      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro21      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro19      CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri1            CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri6            CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
Caleri5            CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
San Teodoro7       CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
La Spezia2         CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
AY509253           CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
µVar               CAGGGAACATAGAATGGTAAAAGTTACGTATAATGAATACGATGAGCAAGAAACGATCAA
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Caleri3            GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri4*           GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro12*     GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro8       GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri7            GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro10      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro14*     GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro17      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro5*      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro11*     GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro4       GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro15      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro1       GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro16      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro21      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro19      GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri1            GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri6            GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
Caleri5            GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
San Teodoro7       GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
La Spezia2         GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
AY509253           GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
µVar               GGTTATTTTCCTCGACAAGAAAGCAACAATAAAAGATCTACATAACCTAATGAGTGTTGG
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Caleri3            TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri4*           TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro12*     TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro8       TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri7            TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro10      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro14*     TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro17      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro5*      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro11*     TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro4       TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro15      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro1       TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro16      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro21      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro19      TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri1            TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri6            TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
Caleri5            TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
San Teodoro7       TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
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La Spezia2          TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT


AY509253            TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
µVar                TAGGGATCTTACAACGGGTGTCTGCAATATAGAAGTACAACCGGAATATGGATTCACACT
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Caleri3             GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri4*            GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro12*      GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro8        GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri7             GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro10       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro14*      GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro17       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro5*       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro11*      GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro4        GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro15       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro1        GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro16       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro21       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro19       GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri1             GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri6             GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
Caleri5             GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
San Teodoro7        GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
La Spezia2          GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAAATGCAGTCTATAGACT
AY509253            GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
µVar                GAGGATACCAGACCCAGACAAGTTGAAATATAAAAGTGATATAGATGCAGTCTATAGACT
                    ******************************************X*****************

Caleri3             CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri4*            CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro12*      CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro8        CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri7             CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro10       CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro14*      CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro17       CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro5*       CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro11*      CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro4        CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro15       CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro1        CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro16       CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro21       CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro19       CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri1             CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri6             CTTTGCTTCAAAATACGACAATAGCGATCTAT
Caleri5             CTTTGCTTCAAAATACGACAATAGCGATCTAT
San Teodoro7        CTTTGCTTCAAAATACGACAATAGCGATCTAT
La Spezia2          CTTCGCTTCAAAATACGACAATAGCGATCTAT
AY509253            CTTCGCTTCAAAATACGACAATAGCGATCTAT
µVar                CTTTGCTTCAAAATACGACAATAGCGATCTAT
                    ***X**************************
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C.7/ Sequence alignment of the OsHV-1 ORFs 35-38, multisite tests, year 2015

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San Teodoro7        ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro16       ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro15       ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro4        ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro5*       ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro17       ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro21       ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
Caleri4*            ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro14*      ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
Caleri5             ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro6        ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
Caleri1             ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
Caleri6             ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro1        ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro11*      ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro9        ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro19       ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
San Teodoro12*      ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
La Spezia2          ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
AY509253            ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
µVar                ATTTCTTCTCTGCCCGTGTCATCGGTGCATATCTTGATCGGCAAGGATTCCTTACTTCC
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San Teodoro7        TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCCGATGTTTCTGATTGTAATTTCTTCT
San Teodoro16       TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCCGATGTTTCTGATTGTAATTTCTTCT
San Teodoro15       TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCCGATGTTTCTGATTGTAATTTCTTCT
San Teodoro4        TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCCGATGTTTCTGATTGTAATTTCTTCT
San Teodoro5*       TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAGTTGTTTCTGATTGTAATTTCTTCT
San Teodoro17       TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAGTTGTTTCTGATTGTAATTTCTTCT
San Teodoro21       TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
Caleri4*            TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro14*      TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
Caleri5             TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro6        TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
Caleri1             TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
Caleri6             TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro1        TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro11*      TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro9        TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro19       TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
San Teodoro12*      TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
La Spezia2          TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
AY509253            TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
µVar                TTGGGACCTCTGATTGGTAGTGAATCAAAATTGCAATTGTTTCTGATTGTAATTTCTTCT
                    **********************************XXX***********************

San Teodoro7        GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro16       GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro15       GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro4        GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro5*       GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro17       GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro21       GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
Caleri4*            GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro14*      GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
Caleri5             GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro         GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
Caleri1             GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
Caleri6             GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro1        GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro11*      GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
48948.A             GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro19       GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
San Teodoro12*      GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
La Spezia2          GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
AY509253            GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
µVar                GTAAGGTTTAGCTTCAGTTTAAGATTGTTTCTCTTTCCACGTCTGTTTCTAATGGGAGCC
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San Teodoro7        ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro16       ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro15       ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro4        ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro5*       ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro17       ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro21       ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
Caleri4*            ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro14*      ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
Caleri5             ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro6        ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
Caleri1             ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
Caleri6             ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro1        ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
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San Teodoro11*     ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro9       ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro19      ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
San Teodoro12*     ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
La Spezia2         ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATAGTCTTTTGTAATATG
AY509253           ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATAGTCTTTTGTAATATG
µVar               ATGGTGATGAATGAAGTTGAAAGACGAAAATCAACAAAATATATA----------------
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San Teodoro7       ------------------------------------------------------------
San Teodoro16      ------------------------------------------------------------
San Teodoro15      ------------------------------------------------------------
San Teodoro4       ------------------------------------------------------------
San Teodoro5*      ------------------------------------------------------------
San Teodoro17      ------------------------------------------------------------
San Teodoro21      ------------------------------------------------------------
Caleri4*           ------------------------------------------------------------
San Teodoro14*     ------------------------------------------------------------
Caleri5            ------------------------------------------------------------
San Teodoro6       ------------------------------------------------------------
Caleri1            ------------------------------------------------------------
Caleri6            ------------------------------------------------------------
San Teodoro1       ------------------------------------------------------------
San Teodoro11*     ------------------------------------------------------------
San Teodoro9       ------------------------------------------------------------
San Teodoro19      ------------------------------------------------------------
San Teodoro12*     ------------------------------------------------------------
La Spezia2         CCGCAGAAAAACTAATAGTGAAAGTAACTTCTTGGAATCGGTCCTCGGAGGATATAAAGT
AY509253           TCGCAGAAAAACTAATAGTGAAAGTAACTTCTTGGAATCGGTCCTCGGAGGATATAAAGT
µVar               ------------------------------------------------------------
                   §XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7       ------------------------------------------------------------
San Teodoro16      ------------------------------------------------------------
San Teodoro15      ------------------------------------------------------------
San Teodoro4       ------------------------------------------------------------
San Teodoro5*      ------------------------------------------------------------
San Teodoro17      ------------------------------------------------------------
San Teodoro21      ------------------------------------------------------------
Caleri4*           ------------------------------------------------------------
San Teodoro14*     ------------------------------------------------------------
Caleri5            ------------------------------------------------------------
San Teodoro6       ------------------------------------------------------------
Caleri1            ------------------------------------------------------------
Caleri6            ------------------------------------------------------------
San Teodoro1       ------------------------------------------------------------
San Teodoro11*     ------------------------------------------------------------
San Teodoro9       ------------------------------------------------------------
San Teodoro19      ------------------------------------------------------------
San Teodoro12*     ------------------------------------------------------------
La Spezia2         TTGACAAAGAGTGCAATGAGGGCTGCCCAAATCACTATCATATTGATGATTCTGAAAAGC
AY509253           TTGACAAAGAGTGCAATGAGGGCTGCCCAAATCACTATCATATTGATGATTCTGAAAAGC
µVar               ------------------------------------------------------------
                   XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7       ------------------------------------------------------------
San Teodoro16      ------------------------------------------------------------
San Teodoro15      ------------------------------------------------------------
San Teodoro4       ------------------------------------------------------------
San Teodoro5*      ------------------------------------------------------------
San Teodoro17      ------------------------------------------------------------
San Teodoro21      ------------------------------------------------------------
Caleri4*           ------------------------------------------------------------
San Teodoro14*     ------------------------------------------------------------
Caleri5            ------------------------------------------------------------
San Teodoro6       ------------------------------------------------------------
Caleri1            ------------------------------------------------------------
Caleri6            ------------------------------------------------------------
San Teodoro1       ------------------------------------------------------------
San Teodoro11*     ------------------------------------------------------------
San Teodoro9       ------------------------------------------------------------
San Teodoro19      ------------------------------------------------------------
San Teodoro12*     ------------------------------------------------------------
La Spezia2         AATAGAACTCTCTGCCATGCCTGTCTCTTTGGTTTCTTCACGATTATGTATTGTGGTTTA
AY509253           AATAGAACTCTCTGCCATGCCTGTCTCTTTGGTTTCTTCACGATTATGTATTGTGGTTTA
µVar               ------------------------------------------------------------
                   XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7       ------------------------------------------------------------
San Teodoro16      ------------------------------------------------------------
San Teodoro15      ------------------------------------------------------------
San Teodoro4       ------------------------------------------------------------
San Teodoro5*      ------------------------------------------------------------
San Teodoro17      ------------------------------------------------------------
San Teodoro21      ------------------------------------------------------------
Caleri4*           ------------------------------------------------------------
San Teodoro14*     ------------------------------------------------------------
Caleri5            ------------------------------------------------------------
San Teodoro6       ------------------------------------------------------------
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Caleri1          ------------------------------------------------------------
Caleri6          ------------------------------------------------------------
San Teodoro1     ------------------------------------------------------------
San Teodoro11*   ------------------------------------------------------------
San Teodoro9     ------------------------------------------------------------
San Teodoro19    ------------------------------------------------------------
San Teodoro12*   ------------------------------------------------------------
La Spezia2       GCAGGGATAAGTTCAGGTTCTTGCTCAATCTCGCACACTGTTTGCTCTGTAGTAGACATA
AY509253         GCAGGGATAAGTTCAGATTCTTGCTCAATCTCGCACACTGTTTGCTCTGTAGTAGACATA
µVar             ------------------------------------------------------------
                 XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7     ------------------------------------------------------------
San Teodoro16    ------------------------------------------------------------
San Teodoro15    ------------------------------------------------------------
San Teodoro4     ------------------------------------------------------------
San Teodoro5*    ------------------------------------------------------------
San Teodoro17    ------------------------------------------------------------
San Teodoro21    ------------------------------------------------------------
Caleri4*         ------------------------------------------------------------
San Teodoro14*   ------------------------------------------------------------
Caleri5          ------------------------------------------------------------
San Teodoro6     ------------------------------------------------------------
Caleri1          ------------------------------------------------------------
Caleri6          ------------------------------------------------------------
San Teodoro1     ------------------------------------------------------------
San Teodoro11*   ------------------------------------------------------------
San Teodoro9     ------------------------------------------------------------
San Teodoro19    ------------------------------------------------------------
San Teodoro12*   ------------------------------------------------------------
La Spezia2       TTGAAAAATGAAAGTGGTTTTCGTAAACTCAAACTTTTTATTTATAGTTTTTTAAAAAAA
AY509253         TTGAAAAATGAAAGTGGTTTTCGTAAACTCAAACTTTT-ATTTATAGTTTTTTAAAAAAA
µVar             ------------------------------------------------------------
                 XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7     ------------------------------------------------------------
San Teodoro16    ------------------------------------------------------------
San Teodoro15    ------------------------------------------------------------
San Teodoro4     ------------------------------------------------------------
San Teodoro5*    ------------------------------------------------------------
San Teodoro17    ------------------------------------------------------------
San Teodoro21    ------------------------------------------------------------
Caleri4*         ------------------------------------------------------------
San Teodoro14*   ------------------------------------------------------------
Caleri5          ------------------------------------------------------------
San Teodoro6     ------------------------------------------------------------
Caleri1          ------------------------------------------------------------
Caleri6          ------------------------------------------------------------
San Teodoro1     ------------------------------------------------------------
San Teodoro11*   ------------------------------------------------------------
San Teodoro9     ------------------------------------------------------------
San Teodoro19    ------------------------------------------------------------
San Teodoro12*   ------------------------------------------------------------
La Spezia2       ACATGGTCCTAGTCAAAATCTCTATAAAAGATGCTAAAATATCCACCAGCCCGCGTTAAG
AY509253         -CATGGTCCTAGTCAAAATCTCTATAAAAGATGCTAAAATATCCACCAGCCCGCGTTAAG
µVar             ------------------------------------------------------------
                 XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7     ------------------------------------------------------------
San Teodoro16    ------------------------------------------------------------
San Teodoro15    ------------------------------------------------------------
San Teodoro4     ------------------------------------------------------------
San Teodoro5*    ------------------------------------------------------------
San Teodoro17    ------------------------------------------------------------
San Teodoro21    ------------------------------------------------------------
Caleri4*         ------------------------------------------------------------
San Teodoro14*   ------------------------------------------------------------
Caleri5          ------------------------------------------------------------
San Teodoro6     ------------------------------------------------------------
Caleri1          ------------------------------------------------------------
Caleri6          ------------------------------------------------------------
San Teodoro1     ------------------------------------------------------------
San Teodoro11*   ------------------------------------------------------------
San Teodoro9     ------------------------------------------------------------
San Teodoro19    ------------------------------------------------------------
San Teodoro12*   ------------------------------------------------------------
La Spezia2       ACATCGGTCTTCACAATACACATGATCCTAGATAATTCCCTGCCAACACTCTTGATCACA
AY509253         ACATTGGTCTCCACAATACACATGATCCTAGATAATTCCCTGCCAACACTCTTGATCACA
µVar             ------------------------------------------------------------
                 XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7     ------------------------------------------------------------
San Teodoro16    ------------------------------------------------------------
San Teodoro15    ------------------------------------------------------------
San Teodoro4     ------------------------------------------------------------
San Teodoro5*    ------------------------------------------------------------
San Teodoro17    ------------------------------------------------------------
San Teodoro21    ------------------------------------------------------------
Caleri4*         ------------------------------------------------------------
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San Teodoro14*  ------------------------------------------------------------
Caleri5         ------------------------------------------------------------
San Teodoro6    ------------------------------------------------------------
Caleri1         ------------------------------------------------------------
Caleri6         ------------------------------------------------------------
San Teodoro1    ------------------------------------------------------------
San Teodoro11*  ------------------------------------------------------------
San Teodoro9    ------------------------------------------------------------
San Teodoro19   ------------------------------------------------------------
San Teodoro12*  ------------------------------------------------------------
La Spezia2      GCTCTTTCTTCCATCGTGATATTATCGGGAGGGAATTGTCGGTTGAGTATCTGTCTATCA
AY509253        GCTCTTTCTTCCATCGTGATATTATCGGGAGGGAATTGTCGGTTGAGTATCTGTCTATCA
µVar            ------------------------------------------------------------
                XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7    ------------------------------------------------------------
San Teodoro16   ------------------------------------------------------------
San Teodoro15   ------------------------------------------------------------
San Teodoro4    ------------------------------------------------------------
San Teodoro5*   ------------------------------------------------------------
San Teodoro17   ------------------------------------------------------------
San Teodoro21   ------------------------------------------------------------
Caleri4*        ------------------------------------------------------------
San Teodoro14*  ------------------------------------------------------------
Caleri5         ------------------------------------------------------------
San Teodoro6    ------------------------------------------------------------
Caleri1         ------------------------------------------------------------
Caleri6         ------------------------------------------------------------
San Teodoro1    ------------------------------------------------------------
San Teodoro11*  ------------------------------------------------------------
San Teodoro9    ------------------------------------------------------------
San Teodoro19   ------------------------------------------------------------
San Teodoro12*  ------------------------------------------------------------
La Spezia2      TTGTGATCGTAAAGGAAATGTGCATTTATCGTGCGATGGTTCCTCGTGAAAAAA-TCATC
AY509253        TTGTGATCGTAAAGGAAATGTGCATTTATCGCGCGATGGTTCCTCGTGAAAAAAATCATC
µVar            ------------------------------------------------------------
                XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

San Teodoro7    ----------------------------------------------------CTATCTT
San Teodoro16   ----------------------------------------------------CTATCTT
San Teodoro15   ----------------------------------------------------CTATCTT
San Teodoro4    ----------------------------------------------------CTATCTT
San Teodoro5*   ----------------------------------------------------CTATCTT
San Teodoro17   ----------------------------------------------------CTATCTT
San Teodoro21   ----------------------------------------------------CTATCTT
Caleri4*        ----------------------------------------------------CTATCTT
San Teodoro14*  ----------------------------------------------------CTATCTT
Caleri5         ----------------------------------------------------CTATCTT
San Teodoro6    ----------------------------------------------------CTATCTT
Caleri1         ----------------------------------------------------CTATCTT
Caleri6         ----------------------------------------------------CTATCTT
San Teodoro1    ----------------------------------------------------CTATCTT
San Teodoro11*  ----------------------------------------------------CTATCTT
San Teodoro9    ----------------------------------------------------CTATCTT
San Teodoro19   ----------------------------------------------------CTATCTT
San Teodoro12*  ----------------------------------------------------CTATCTT
La Spezia2      AAATTGTTCTAATACTTCGGGGCTGAACGGTGGTACATTGGTTACATCTTTTACTATCTT
AY509253        AAATTGTTCTAATACTTCGGGGCTGAACGGTGGTACATTGGTTACATCTTTTACTATCTT
µVar            ----------------------------------------------------CTATCTT
                XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX*******

San Teodoro7    TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro16   TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro15   TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro4    TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro5*   TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro17   TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro21   TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
Caleri4*        TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro14*  TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
Caleri5         TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro6    TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
Caleri1         TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
Caleri6         TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro1    TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro11*  TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro9    TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro19   TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
San Teodoro12*  TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
La Spezia2      TTTGGCATTGATGATTACGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
AY509253        TTTGGCATTGATGATTATGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
µVar            TTTGGCATTGATGATTATGCTTTTGAGTATCGTCCACAAGTACCTTGTATGTGGTATATC
                ****************X*****************************************

San Teodoro7    TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro16   TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro15   TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro4    TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro5*   TTCCCATAATGGATATTCCGTGTTTACAG
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San Teodoro17       TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro21       TTCCCATAATGGATATTCCGTGTTTACAG
Caleri4*            TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro14*      TTCCCATAATGGATATTCCGTGTTTACAG
Caleri5             TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro6        TTCCCATAATGGATATTCCGTGTTTACAG
Caleri1             TTCCCATAATGGATATTCCGTGTTTACAG
Caleri6             TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro1        TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro11*      TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro9        TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro19       TTCCCATAATGGATATTCCGTGTTTACAG
San Teodoro12*      TTCCCATAATGGATATTCCGTGTTTACAG
La Spezia2          TTCCCATAATGGATATTCCGTGTTTACAG
AY509253            TTCCCATAATGGATATTCCATGTTTACAG
µVar                TTCCCATAATGGATATTCCATGTTTACAG
                    ******************x*********
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C.8/ Sequence alignments of ORFs of the OsHV-1 isolated in Normandy in 2016 with OsHV-1 isolated in previous years

ORF 118

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NORMANDY        ATGAATAGCAAATTGAATAGTGCTATGTATAGTGCGCATATGATAATGCGTCACGCATTT
KF517165.1      ATGAATAGCAAATTGAATAGTGCTATGTATAGTGCGCATATGATAATGCGTCACGCATTT
                ************************************************************

NORMANDY        GGATATAATGACTACAACAACAAGTATGGGCGTATCAATGACGTATATAACAAGATGGCG
KF517165.1      GGATATAATGACTACAACAACAAGTATGGGCGTATCAATGACGTATATAACAAGATGGCG
                ************************************************************

NORMANDY        GATGGCAAGAGACTACGATTGGAGGAGAGAGAGGTCAAGAGTTTACGTGGGTTGGTCTGC
KF517165.1      GATGGCAAGAGACTACGATTGGAGGAGAGAGAGGTCAAGAGTTTACGTGGGTTGGTCTGC
                ************************************************************

NORMANDY        ACACTTAAAATGATGATTAAAAACACTGACATAATTACGTATGACGAGGAGTGCTGTATC
KF517165.1      ACACTTAAAATGATGATTAAAAACACTGACATAATTACGTATGACGAGGAGTGCTGTATC
                ************************************************************

NORMANDY        TGTATGGCTAAAAACAACAGGAAGGAGGCGCTTCCCTGCCAACATAATGTATGCAGAGAC
KF517165.1      TGTATGGCTAAAAACAACAGGAAGGAGGCGCTTCCCTGCCAACATAATGTATGCAGAGAC
                ************************************************************

NORMANDY        TGTTATTATAAGCCCATGCGCAATAACTGCCCTGTTTGCAATATGGAATGGCCAATGAGA
KF517165.1      TGTTATTATAAGCCCATGCGCAATAACTGCCCTGTTTGCAATATGGAATGGCCAATGAGA
                ************************************************************

NORMANDY        AAGGACGATAAACACGCTGCTCCATATGGATTGGCTGAATACGCACACACCTACGGAGGA
KF517165.1      AAGGACGATAAACACGCTGCTCCATATGGATTGGCTGAATACGCACACACCTACGGAGGA
                ************************************************************

NORMANDY        GAGGAGCAAAGAACGCCTTCGCCACCCGTATTAGGAACTGTGGAGGGAGGTGACATTTCT
KF517165.1      GAGGAGCAAAGAACACCTTCGCCACCCGTATTAGGAACTGTGGAGGGAGGTGACATTTCT
                ************** ************************************************

NORMANDY        CCTCGATTGGTCGGCGCAATTAGAACTAACGACACGTGGTTATCTTCAAGGCGGGATAGT
KF517165.1      CCTCGATTGGTCGGCGCAATTAGAACTAACGACACGTGGTTATCTTCAAGGCGGGATAGT
                ************************************************************

NORMANDY        CCATACCATATAGAGAACAGGATACACAATAATAACAACAACAACTATGACGAAAATAAC
KF517165.1      CCATACCATATAGAGAACAGGATACACAATAATAACAACAACAACTATGACGAAAATAAC
                ************************************************************

NORMANDY        CCTGACGACCTTCCGGTAATACACCCACCCAGAAGACGTCATCGGCAAACTGCGCACATA
KF517165.1      CCTGACGACCTTCCGGTAATACACCCACCCAGAAGACGTCATCGGCAAACTGCGCACATA
                ************************************************************

NORMANDY        TCCATATAA
KF517165.1      TCCATATAA
                *********
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ORF 5

```
NORM            ATGGGGTTTTTGTTACGTTATTGATTAGCGCATTATTGATTATTGTGCACGCAGACCCAC
KF517186.1      ATGGGGTTTTTGTTACGTTATTGATTAGCGCATTATTGATTATTGTGCACGCAGACCCAC
                ************************************************************

NORM            CCATTATTAATGTCGAAGTCAATCCTTATGGTATTATATCTGTCACATGTTCGGCAATAC
KF517186.1      CCATTATTAATGTCGAAGTCAATCCTTATGGTATTATATCTGTCACATGTTCGGCAATAC
                ************************************************************

NORM            ATCCTAGACTGTACAAAATTAGAATGTACAACAATAAGGTCAAGGGTGATGCAACCATAA
KF517186.1      ATCCTAGACTGTACAAAATTAGAATGTACAACAATAAGGTCAAGGGTGATGCAACCATAA
                ************************************************************

NORM            GTTTTTACGCGCATATAAATCTATCACCATGTGTTGTGGATTCCAGCGGCGATTCATCAT
KF517186.1      GTTTTTACGCGCATATAAATCTATCACCATGTGTTGTGGATTCCAGCGGCGATTCATCAT
                ************************************************************

NORM            TATACGATAGTGTTTCATGTTCTATTCAGGGAAGTAAGGGGTCAGTGACTGTGGTATACA
KF517186.1      TATACGATAGTGTTTCATGTTCTATTCAGGGAAGTAAGGGGTCAGTGACTGTGGTATACA
                ************************************************************

NORM            ACCGCGATGTCAAAGAAGAAATGTTGGGAGAATGGGCATGTATGGAATATGATCCTTTAG
KF517186.1      ACCGCGATGTCAAAGAAGAAATGTTGGGAGAATGGGCATGTATGGAATATGATCCTTTAG
                ************************************************************

NORM            ATCAACCTTTAGAATATGAAGAGTACAACAGGGAACTATACGACTTATATGCTGTCAAGA
KF517186.1      ATCAACCTTTAGAATATGAAGAGTACAACAGGGAACTATACGACTTATATGCTGTCAAGA
                ************************************************************

NORM            ATGCTTATTATGATGACAACATTACCGCGTTATCACCTCCCAACAGTCTGTCAATAGAAA
KF517186.1      ATGCTTATTATGATGACAACATTACCGCGTTATCACCTCCCAACAGTCTGTCAATAGAAA
                ************************************************************

NORM            TTATGGATTATAATTACAGAGTTGGTAACACTGGGTTGTAAATCAAAAATACCCATAGAA
KF517186.1      TTATGGATTATAATTACAGAGTGGGTAACACTGGGTTGTAAATCAAAAATACCCATAGAA
                ********************* ************************************

NORM            CTTCCGGGAAGCACCGTAATAGCACCACCCAATAAAGATATATTTCTTATCTCTGACCAT
KF517186.1      CTTCCGGGAAGCACCGTAATAGCACCACCCAATAAAGATATATTTCTTATCTCTGACCAT
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                  ************************************************************
NORM              GTGTTTCTATCGCGAGGTGATTTACTGGGCAGGAAATCATATTACTCTACGAGGAAAATT
KF517186.1        GTGTTTCTATCGCGAGGTGATTTACTGGGCAGGAAATCATATTACTCTACGAGGGAAATT
                  ************************************************************

NORM              TGCGAGAGAAGAGATTATAACAATATAGTTTTAGAATGTTATGGCGTGGAGGAATCAGGA
KF517186.1        TGCGAGAGAAGAGATTATAACAATATAGTTTTAGAATGTTATGGCGTGGAGGAATCAGGA
                  ************************************************************

NORM              ACAGAATTCAATTACATCAAACCCACTTTTCCTATGATTATTAGCAGTGATCTTAGCGGT
KF517186.1        ACAGAATTCAATTACATCAAACCCACTTTTCCTATGATTATTAGCAGTGATCTTAGCGGT
                  ************************************************************

NORM              GTTTTCATGAGTGATAATAAAATCGTGGTGGATAAAACGATAATGTTTCCTACGTCTGCC
KF517186.1        GTTTTCATGAGTGATAATAAAATCGTGGTGGATAAAACGATAATGTTTCCTACGTCTGCC
                  ************************************************************

NORM              GGTGATTCTATAAGCCTATCGGGAGATCTCGTTGTTATCGAATTACCCAGAGAGGAAGAT
KF517186.1        GGTGATTCTATAAGCCTATCGGGAGATCTCGTTGTTATCGAATTACCCAGAGAGGAAGAT
                  ************************************************************

NORM              TACATGGAAATAGAAATGTACAATGGTGTTCACTTCAATCCAAACTCAGAACACCTTACA
KF517186.1        TACATGGAAATAGAAATGTACAATGGTGTTCACTTCAATCCAAACTCAGAACACCTTACA
                  ************************************************************

NORM              AACATGGCCAAAATCTTAATAGAACATTTGTCTAGTTCTTCAACCTTTCAGATCAGGGAT
KF517186.1        AACATGGCCAAAATCTTAATAGAACATTTGTCTAGTTCTTCAACCTTTCAGATCAGGGAT
                  ************************************************************

NORM              TTAAATGAGACGTATGACGAATTAGATGATGTGGATATGCACTATATATTTTACGGGAAG
KF517186.1        TTAAATGAGACGTATGACGAATTAGATGATGTGGATATGCACTATATATTTTACGGGAAG
                  ************************************************************

NORM              AAGGAATGTCTACCTAGATTTAAAAAAAATAATTTTAAACAACTGTAGGAGTTCCGGTGA
KF517186.1        AAGGAATGTCTACCTAGATTTAAAAAAAATAATTTTAAACAACTGTAGGAGTTCCGGTGA
                  ************************************************************

NORM              GATATCCGTGATAGATTCTATGAATCTTCATATGTTATTCGATTGCAATGATATTTCTCG
KF517186.1        GATATCCGTGATAGATTCTATGAATCTTCATATGTTATCCGATTGCAATGATATTTCTCG
                  ************************************ ***********************

NORM              ATTTTATTACGCCTTTGCAATCAATTCAACATTATCTCAACCAATCAATAGAGAGGTGGA
KF517186.1        ATTTTATTACGCCTTTGCAATCAATTCAACATTATCTCAACCAATCAATAGAGAGGTGGA
                  ************************************************************

NORM              AATAATACCCATCAATCTACCAAGAACAGATACACATAGTATCACATTACCACCCGATGA
KF517186.1        AATAATACCCATCAATCTACCAAGAACAGATACACATAGTATCACATTACCACCCGATGA
                  ************************************************************

NORM              TTACCCAGATTCCCCTCGAGGTAGCTTTTGTCAAGATGAAACAAAATATTATGCAACGAG
KF517186.1        TTACCCAGATTCCCCTCGAGGTAGCTTTTGTCAAGATGAAACAAAATATTATGCAACGAG
                  ************************************************************

NORM              GAAATTATACGTCAACATACAACATCACTGTGAATACAACGGAGATTTGACAAGGTGTTC
KF517186.1        GAAATTATACGTCAACATACAACATCACTGTGAATACAACGGAGATTTGACAAGGTGTTC
                  ************************************************************

NORM              TATACCATACGACCTACACAAACCTGTTTTTGAAAACAGGTGTGAAAAGAAACCAAATGT
KF517186.1        TATACCATACGACCTACACAAACCTGTTTTTGAAAACAGGTGTGAAGAGAAACCAAATGT
                  ********************************************** **************

NORM              GTGTTATCCCAATGCACTCTTTACCATGAAGATACCCACCAATGTGGTAAAGACGGAACA
KF517186.1        GTGTTATCCCAATGCACTCTTTACCATGAAGATACCCACCAATGTGGTAAAGACGGAACA
                  ************************************************************

NORM              ATCTTTTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTG
KF517186.1        ATCTTTTTCTAGGATATGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTG
                  ************************************************************

NORM              GGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGA
KF517186.1        GGATAATGATTTTAGAATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGA
                  ************************************************************

NORM              GCTATTACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCC
KF517186.1        GCTATTACCCGACCACAAACCTAACGTTGTATTCGATTACGGATTAAGAAAATGGGTTCC
                  ************************************************************

NORM              ACAATCTAAAATTAAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAG
KF517186.1        ACAATCTAAAATTAAAAACCCACATGGGGGCCAAGGAATTTAAAGCCCCGGGGAAAAAAG
                  ************************************************************

NORM              TATAA
KF517186.1        TATAA
                  *****
```

ORF 25

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NORM              CTAATGTAAATATACCCTTTTCAGGTTGGCAATGGTGATAAAAATAGGAAATATTACAAG
KF517271.1        CTAATGTAAATATACCCTTCTCAGGTTGGCAATGGTGATAAAAATAGGAAATATTACAAG
                  ****************** ****************************************

NORM              AATGATGGCACATATCCCTGATATGACTGATACAGACATTGGAACGCCTCTGGCTTTCGC
KF517271.1        AATGATGGCACATATCCCTGATATGACTGATACAGACATTGGAACACCTCTGGCTTTCGC
                  ****************************************** ***************

NORM              CAATGTGTCATTCAGTAATTTATCCACTGTTTGTGAATTTGTGGAAGGTCTAGACACGAC
KF517271.1        CAATGTGTCATTCAGTAATTTATCCACTGTTTGTGAATTTGTGGAAGGTCTAGACACGAC
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                  ************************************************************
NORM              ATTGTCTGTTGATTGTCTTGGAAAATTTACATTGAAGTTGACCAGGTCTAATCCCTTGAT
KF517271.1        ATTGTCTGTTGATTGTCTTGGAAAATTTACATTGAAGTTGACCAGGTCTAATCCCTTGAT
                  ************************************************************

NORM              TTCTGCCACATTCCCCTTTGCCGGTGTTATCACCTTTTCCCAAATTAAATTACATGCTAT
KF517271.1        TTCTGCCACATTCCCCTTTGCCGGTGTTATCACCTTTTCCCAAATTAAATTACATGCTAT
                  ************************************************************

NORM              AGGTCCATGTGAAATCATCCTGGAAATGTGTTCTGGTGTTGATTCTATTTTAACTCGCCA
KF517271.1        AGGTCCATGTGAAATCATCCTGGAAATGTGTTCTGGTGTTGATTCTATTTTAACTCGCCA
                  ************************************************************

NORM              AAGGTCGTATCCATCCGTGGTCGTGCCTGTATCTGTAAACTGATTCATTATACTTCTTTT
KF517271.1        AAGGTCGTATCCATCCGTGGTCGTGCCTGTATCTGTAAACTGATTCATTATACTTCTTTT
                  ************************************************************

NORM              ATTACCAAGGCAATATACTGCCTTTACATCACCTCCGCTATTCTTTTTAACATATAGATC
KF517271.1        ATTACCAAGGCAATATACTGCCTTTACATCACCTCCGCTATTCTTTTTAACATATAGATC
                  ************************************************************

NORM              AAGTACTGTTTCATCCCCTGCGTATTGTACCGGTGTTTGAAACAATCCTTTAACATGGAA
KF517271.1        AAGTACTGTTTCATCCCCTGCGTATTGTACCGGTGTTTGAAACAATCCTTTAACATGGAA
                  ************************************************************

NORM              TTCACCCTTGTGGTGTAATGACGTCAGAGTCTCATGATATATATTTGAGTGATCCTGGCT
KF517271.1        TTCACCCTTGTGGTGTAATGACGTCAGAGTCTCATGATATATATTTGAGTGATCCTGGCT
                  ************************************************************

NORM              GGTCGATTCATTTACGAAGCAGAGTGCCACATAGACAAGAACTATTAACTTAGCAGCTAA
KF517271.1        GGTCGATTCATTTACGAAGCAGAGTGCCACATAGACAAGAACTATTAACTTAGCAGCTAA
                  ************************************************************

NORM              AGTCAT
KF517271.1        AGTCAT
                  ******
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ORF 32

```
NORM              ATGACGCCAATTAAACTACTCTTAACCCTGTTGTTAATTAACGGGGTTAGATCTGCAATG
KF517250.1        ATGACGCCAATTAAACTACTCTTAACCCTGTTGTTAATTAACGGGGTTAGATCTGCAATG
                  ************************************************************

NORM              TTAGCATTTAAAATTTTTAGAAATGGATGACAAAACAACTCTATTACTGGACAGTTCAAT
KF517250.1        TTAGCATTTAAAATTTTTAGAAATGGATGACAAAACAACTCTATTACTGGACAGTTCAAT
                  ************************************************************

NORM              TAGTAATAGTAAACACACCATGGTCAGCACCTCATATCCAAACCTGAATTTTAATTTTAC
KF517250.1        TAGTAATAGTAAACACACCATGGTCAGCACCTCATATCCAAACCTGAATTTTAATTTTAC
                  ************************************************************

NORM              TACAACGACAGAATGTGCGACAGAGCCGCCTGATTTGGAATCATCGTGTACAAAAGATTT
KF517250.1        TACAACGACAGAATGTGCGACAGAGCCGCCTGATTTGGAATCATCGTGTACAAAAGATTT
                  ************************************************************

NORM              TTAGAGGTGTTTGGAGAGTTCAAATTGAAGTACCAAACACGGTAAACCTTGTTAATAAAT
KF517250.1        TTAGAGGTGTTTGGAGAGTTCAAATTGAAGTACCAAACACGGTAAACCTTGTTAATAAAT
                  ************************************************************

NORM              ATAACGCGTATTGGGGAATGTGGAGAGCCACGATTTTATTAACAAATAAGGTTGCAGAAT
KF517250.1        ATAACGCGTATTGGGGAATGTGGAGAGCCACGATTTTATTAACAAATAAGGTTGCAGAAT
                  ************************************************************

NORM              GGACAGGTAGCAGAACATTAAAATTCGTCAAAGATTTTAAGTCAAACGAATATCGCCGGG
KF517250.1        GGACAGGTAGCAGAACATTAAAATTCGTCAAAGATTTTAAGTCAAACGAATATCGCCGGG
                  ************************************************************

NORM              GAAAACAGAAGTGAAAAAGATCAGCAACACAGCGGATAAACTCATAATAGGCTGTGGCAC
KF517250.1        GAAAACAGAAGTGAAAAAGATCAGCAACACAGCGGATAAACTCATAATAGGCTGTGGCAC
                  ************************************************************

NORM              ACCTGGTTTAGATTTACGCAGAACATTGACATATGCTAGACCAGACAGTTTAATCATGGA
KF517250.1        ACCTGGTTTAGATTTACGCAGAACATTGACATATGCTAGACCAGACAGTTTAATCATGGA
                  ************************************************************

NORM              ATACTATGTAAGACCTCATGTGTCCATCTATAGAGATGGTGTTAAACTGCTAGAAGATGA
KF517250.1        ATACTATGTAAGACCTCATGTGTCCATCTATAGAGATGGTGTTAAACTGCTAGAAGATGA
                  ************************************************************

NORM              TATATTCGCAAGATATAGAAAGACGGAATCTGTCACAAAGATGTTAAGAGATTGTGCCAT
KF517250.1        TATATTCGCAAGATATAGAAAGACGGAATCTGTCACAAAGATGTTAAGAGATTGTGCCAT
                  ************************************************************

NORM              GGGCGCCAAACCTCCCATAATCGAACAGACCAATCCTAAATGTAGTGGAGCCGCGGTCAT
KF517250.1        GGGCGCCAAAACTCCCATAATCGAACAGACCAATCCTAAATGTAGTGGAGCCGCGGTCAT
                  ********** ***************************************************

NORM              CGGTAATAACCCAGTATATGAAAATAAAATAGAACCAATGCCAAACCGATGGGTAAAATA
KF517250.1        CGGTAATAACCCAGTATATGAAAATAAAATAGAACCAATGCCAAACCGATGGGTAAAATA
                  ************************************************************

NORM              TGGTTGGGAGCTACCGACTAGCGATCCACCAGATTTCCCAGTAGGAGGAATATATTATAA
KF517250.1        TGGTTGGGAGCTACCGACTAGCGATCCACCAGATTTCCCAGTAGGAGGGATATATTATAA
                  ********************************************** ************

NORM              CTTGGGCAGTACGATATTAAATATAATAAGCCCAGATTATACTTCCAATGAATTCACACA
KF517250.1        CTTGGGCAGTACGATATTAAATATAATAAGCCCAGATTATACTTCCAATGAATTCACACA
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                ************************************************************
NORM            CCTGTCTCCTCATGTTTATAAACATAGTTTTAGATTGTTCATATACTACACCCTGAAATA
KF517250.1      CCTGTCTCCTCATGTTTATAAACATAGTTTTAGATTGTTCATATACTACACCCTGAAATA
                ************************************************************

NORM            CACCCGGGTTAAAATGGTGAAAGGACCGTCGGTAAATATTGACGAGTTCAAAGGTGGAAG
KF517250.1      CACCCGGGTTAAAATGGTGAAAGGACCGTCGGTAAATATTGACGAGTTCAAAGGTGGAAG
                ************************************************************

NORM            AGTGGGTGTGTTAGCTTTGAGGGATACAAGAAGGATAAAACCATGGCTTACCTCCTTTAC
KF517250.1      AGTGGGTGTGTTAGCTTTGAGGGATACAAGAAGGATAAAACCATGGCTTACCTCCTTTAC
                ************************************************************

NORM            AAACGTCAATAATTGTAAAATATCAGCCATAACAGGAGAGGTGAATATTCAGTCCTCTGA
KF517250.1      AAACGTCAATAATTGTAAAATATCAGCCATAACAGGAGAGGTGAATATTCAGTCCTCTGA
                ************************************************************

NORM            GCCATCAACAAAAATATTTTGGGATGTTGTTCCGGAAGACCAGGGAAATGTTGGCTCAGA
KF517250.1      GCCATCAACAAAAATATTTTGGGATGTTGTTCCGGAAGACCAGGGAAATGTTGGCTCAGA
                ************************************************************

NORM            TTATATTTATATATTCTATAATCCCAGGAGCGGCGCGGTAGGGAGAATTAATGGAATGGA
KF517250.1      TTATATTTATATATTCTATAATCCCAGGAGCGGCGCGGTAGGGAGAATTAATGGAATGGA
                ************************************************************

NORM            ATATAAAGTATTTCCAGAGGCTGGAACCGATGAAACTACGTTAGATAAGACCATTACCCA
KF517250.1      ATATAAAGTATTTCCAGAGGCTGGAACCGATGAAACTACGTTAGATAAGACCATTACCCA
                ************************************************************

NORM            AACAGACTCAACATGTAATTTACAGATTACCGCAGTGAAAAGCAAGGATTACCAGGATGG
KF517250.1      AACAGACTCAACATGTAATTTACAGATTACCGCAGTGAAAAGCAAGGATTACCAGGATGG
                ************************************************************

NORM            ATATTACGACATGCTTAAGACGTATGTTTTACCAAAGAGTTCAAAACTTCACGAGGATTT
KF517250.1      ATATTACGACATGCTTAAGACGTATGTTTTACCAAAGAGTTCAAAACTTCACGAGGATTT
                ************************************************************

NORM            CCGGTCAGATGACTATCAGTCGGTATTCCAATATATCACTCCCGAAAACACATGTGGTGA
KF517250.1      CCGGTCAGATGACTATCAGTCGGTATTCCAATATATCACTCCCGAAAACACATGTGGTGA
                ************************************************************

NORM            TAAACAAAGAGCAAATAGATGTGGCACGGTTAATCCAGTGACAGATAAAGTGTTTTCTAC
KF517250.1      TAAACAAAGAGCAAATAGATGTGGCACGGTTAATCCAGTGACAGATAAAGTGTTTTCTAC
                ************************************************************

NORM            TGTGGAATTTGAGGTCGACAAGGTGGATGGAAATTACACTTGCATATTTGCCGGAGACCC
KF517250.1      TGTGGAATTTGAGGTCGACAAGGTGGATGGAAATTACACTTGCATATTTGCCGGAGACCC
                ************************************************************

NORM            CAGCAAAAATTTTTATTACGAGGGTGTTATTCCTACAACCACAACCACTACTACAACTAC
KF517250.1      CAGCAAAAATTTTTATTACGAGGGTGTTATTCCTACAACCACAACCACTACTACAACTAC
                ************************************************************

NORM            AACTCCTGCTCCTACAACCACTACTACAACTACCACCACTCCTGCTCCTACAACCACTAC
KF517250.1      AACTCCTGCTCCTACAACCACTACTACAACTACCACCACTCCTGCTCCTACAACCACTAC
                ************************************************************

NORM            TACAACCACTACAACACCTGTTCCTACCACTACTACCACTACCCCTGCTCCTACAACCAC
KF517250.1      TACAACCACTACAACACCTGTTCCTACCACTACTACCACTACCCCTGCTCCTACAACCAC
                ************************************************************

NORM            CACAACAACCACTACACTTGCTCCTACAACGACGACGCCAACTACAACCACTACTACCAC
KF517250.1      CACAACAACCACTACACTTGCTCCTACAACGACGACGCCAACTACAACCACTACTACCAC
                ************************************************************

NORM            CATTCCCACAACAACCACACCGATTATTTTAAAACCACCAACGAAAAACCCATTGATTAT
KF517250.1      CATTCCCACAACAACCACACCGATTATTTTAAAACCACCAACGAAAAACCCATTGATTAT
                ************************************************************

NORM            TGATCAGGATAAGAATGTTACCAGTACAACGACACCCAAGGAATTGGAATCAAATAAAGA
KF517250.1      TGATCAGGATAAGAATGTTACCAGTACAACGACACCCAAGGAATTGGAATCAAATAAAGA
                ************************************************************

NORM            TACCATATTCGTCAAAATTAAAGATGTGGTATTTTCATATAAAATAAAAAGTACACCTGA
KF517250.1      TACCATATTCGTCAAAATTAAAGATGTGGTATTTTCATATAAAATAAAAAGTACACCTGA
                ************************************************************

NORM            TGACCATAGACGATACAGGAATTATTCACAACAGGAGATTCCAAAAATAAACTTAGACTG
KF517250.1      TGACCATAGACGATACAGGAATTATTCACAACAGGAGATTCCAAAAATAAACTTAGACTG
                ************************************************************

NORM            GTTATTACTTTATATGGCCGCTCTTGGCGGTAGTTTATTTGTATCATTCATAATAATATG
KF517250.1      GTTATTACTTTATATGGCCGCTCTTGGCGGTAGTTTATTTGTATCATTCATAATAATATG
                ************************************************************

NORM            TTCTATATGCATTTACATTAGAAAAATGAAATAA
KF517250.1      TTCTATATGCATTTACATTAGAAAAATGAAATAA
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NORM            ATGACAAACATGATTTTATTATCGGCAGTGTTTCTATCACTAGCTATTTTAGAAACACAT
KF517285.1      ATGACAAACATGATTTTATTATCGGCAGTGTTTCTATCACTAGCTATTTTAGAAACACAT
                ************************************************************

NORM            TGTGCTAATCATATAACAACAGGTATATCAACCGCTGGAGAGGTGGAAATTAGGTGTGTG
KF517285.1      TGTGCTAATCATATAACAACAGGTATATCAACCGCTGGAGAGGTGGAAATTAGGTGTGTG
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                ************************************************************
NORM            TCAGATAACTATGTAAAGAAAATAATGGCGTATAACAGGGATCGTTTATCTGACGCCACT
KF517285.1      TCAGATAACTATGTAAAGAAAATAATGGCGTATAACAGGGATCGTTTATCTGACGCCACT
                ************************************************************

NORM            ATCAGTATAATACAAAACTATAAACAAGGCCCATATGGGTGTGATGGTGTAGCGCAAAGT
KF517285.1      ATCAGTATAATACAAAACTATAAACAAGGCCCATATGGGTGTGATGGTGTAGCGCAAAGT
                ************************************************************

NORM            AGTGTTATATCCGGTGCCGCAAAGGAGAAACGGATAAATACCCCAGAATGTATCACGACC
KF517285.1      AGTGTTATATCCGGTGCCGCAAAGGAGAAACGGATAAATACCCCAGAATGTATCACGACC
                ************************************************************

NORM            GGGAGGAGAATAGGCGGGCTAATTGTTAGACTGAAAAGTAAACCAAAAGAAGAAGATCTT
KF517285.1      GGGAGGAGAATAGGCGGGCTAATTGTTAGACTGAAAAGTAAACCAAAAGAAGAAGATCTT
                ************************************************************

NORM            GGTAAATGGGAATGTTATTTTACGGATCTTACTGATAATGTTGATTCAAAAACCTTAACC
KF517285.1      GGTAAATGGGAATGTTATTTTACGGATCTTACTGATAATGTTGATTCAAAAACCTTAACC
                ************************************************************

NORM            GATGTGTTAACCAAATATAACCTTCCCAACAGTGGTTATTTTAACGCGGATGGTACTCCC
KF517285.1      GATGTGTTAACCAAATATAACCTTCCCAACAGTGGTTATTTTAACGCGGATGGTACTCCC
                ************************************************************

NORM            AATGACTTTACTACGGAATTAAAGGTTTTGGATTACGACGATGTAACAAAAGACGTAATC
KF517285.1      AATGACTTTACTACGGAATTAAAGGTTTTGGATTACGACGATGTAACAAAAGACGTAATC
                ************************************************************

NORM            ATGGGATGTAAACTTAGCACCGATTTAGGCACACCTCCCACCATGTCAGAAAAACAACCA
KF517285.1      ATGGGATGTAAACTTAGCACCGATTTAGGCACACCTCCCACCATGTCAGAAAAACAACCA
                ************************************************************

NORM            TACAGCAGTGTACACCTTACAGGAGATGATAGATATTATGCTGAGGGTGATTTATACAGC
KF517285.1      TACAGAAGTGTACACCTTACAGGAGATGATAGATATTATGCTGAGGGTGATTTATACAGC
                ***** ********************************************************

NORM            AGAACGTCATTTAGTTCTACACCTTACGATTGCACAGGGATACCACAGAACACAAATAAA
KF517285.1      AGAACGTCATTTAGTTCTACACCTTACGATTGCACAGGGATACCACAGAACACAAATAAA
                ************************************************************

NORM            TATCTATACAATTGTTTTAACGTTGCCCCCAATGAATATAGGTCGGCCGCTCACAGACCT
KF517285.1      TATCTATACAATTGTTTTAACGTTGCCCCCAATGAATATAGGTCGGCCGCTCACAGACCT
                ************************************************************

NORM            CCACCCTTCATCGCCTTAACAGCACCATCCACACCATCCAATATCATTTTGGATAATGTA
KF517285.1      CCACCCTTCATCGCCTTAACAGCACCATCCACACCATCCAATATCATTTTGGATAATGTA
                ************************************************************

NORM            GCAGAAAAACATGCGTTTACAAAGCATTTCTTTTTCCCTGCCGAAGATAAGGCGGTGGTA
KF517285.1      GCAGAAAAACATGCGTTTACAAAGCATTTCTTTTTCCCTGCCGAAGATAAGGCGGTGGTA
                ************************************************************

NORM            GATTTACGAGGTAAAAATGTCGTAATATCACCCCTGGCAGTTTCAGACTACACACAGATA
KF517285.1      GATTTACGAGGTAAAAATGTCGTAATATCACCCCTGGCAGTTTCAGACTACACACAGATA
                ************************************************************

NORM            AAGATGAAATATCTTTCTGTTCAAAACGCCGTATTTTCACCTCCAGAAGAACTAATTAAT
KF517285.1      AAGATGAAATATCTTTCTGTTCAAAACGCCGTATTTTCACCTCCAGAAGAACTAATTAAT
                ************************************************************

NORM            AAGCTTGGTGAAGTGTTACTCAAGGAATTATCCACAGCCACCAAATTTGAAATTGTCAAT
KF517285.1      AAGCTTGGTGAAGTGTTACTCAAGGAATTATCCACAGCCACCAAATTTGAAATTGTCAAT
                ************************************************************

NORM            GTTGACAATAATTTCGCGCCAACTATACCAGGGGACCCCTATAAAATGGTAATGATAGCA
KF517285.1      GTTGACAATAATTTCGCGCCAACTATACCAGGGGACCCCTATAAAATGGTAATGATAGCA
                ************************************************************

NORM            GACCAATCATGTGTGAGCGGTGTGAATAAAAATGTAATATACACCGGATTATGCAAGAAA
KF517285.1      GACCAATCATGTGTGAGCGGTGTGAATAAAAATGTAATATACACCGGATTATGCAAGAAA
                ************************************************************

NORM            TCATCGTCGATGTCTGTCATAACCATGACTTATTTCTCATCGTTGGATTGCGACAAGACC
KF517285.1      TCATCGTCGATGTCTGTCATAACCATGACTTATTTCTCATCGTTGGATTGCGACAAGACC
                ************************************************************

NORM            TCACATTATGCATATATATTTTACACCGGTAATGAGCCTAGAGAGGTTGAAATTCTACCA
KF517285.1      TCACATTATGCATATATATTTTACACCGGTAATGAGCCTAGAGAGGTTGAAATTCTACCA
                ************************************************************

NORM            GCTGGCATACAAGAAGCAGTTGGTACACCACCAGATGTCACAGCTAGCAACATCCCCGAA
KF517285.1      GCTGGCATACAAGAAGCAGTTGGTACACCACCAGATGTCACAGCTAGCAACATCCCCGAA
                ************************************************************

NORM            AATTCTGGCGGTAATGACTGTGAATCTACGACAGCCCAGGATGTAGCAGAAAAACTTTTC
KF517285.1      AATTCTGGCGGTAATGACTGTGAATCTACGACAGCCCAGGATGTAGCAGAAAAACTTTTC
                ************************************************************

NORM            GGTCACTTGCAAACCGTGTGTCCCACCATTTCCCTGTGTCAAATGCCATCTACTGTGAGA
KF517285.1      GGTCACTTGCAAACCGTGTGTCCCACCATTTCCCTGTGTCAAATGCCATCTACTGTGAGA
                ************************************************************

NORM            ACAGTCATTACTGACGGGGAATGCGCTCAGAGACCATTTATCTGTAACAATAGGGCATTG
KF517285.1      ACAGTCATTACTGACGGGGAATGCGCTCAGAGACCATTTATCTGTAACAATAGGGCATTG
                ************************************************************

NORM            GTTGAATATCATGTACCACTAACAGAGTTAACATCTGGTTCCCCTTTCGCCAACGATTGG
KF517285.1      GTTGAATATCATGTACCACTAACAGAGTTAACATCTGGTTCCCCTTTCGCCAACGATTGG
                ************************************************************

NORM            AGTTGCACTGCGGTGGATAAACAAAGTCCAATGAAGACATGGCACAAGGGATTGAGAACG
KF517285.1      AGTTGCACTGCGGTGGATAAACAAAGTCCAATGAAGACATGGCACAAGGGATTGAGAACG
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                ************************************************************
NORM            GAATTGGCATGTGGTTTGGGTGATTTAAAACAGAAATACATTGACAATTCATTGCCATTG
KF517285.1      GAATTGGCATGTGGTTTGGGTGATTTAAAACAGAAATACATTGACAATTCATTGCCATTG
                ************************************************************

NORM            ATAGTAAAGCAGGGTAAAGATTCCTACAAAGTGGTTTGCTCCACACCCCCATCATTGTGC
KF517285.1      ATAGTAAAGCAGGGTAAAGATTCCTACAAAGTGGTTTGCTCCACACCCCCATCATTGTGC
                ************************************************************

NORM            ACCGACAATGGTCTAACTCCACCCAGATTAAATAAAGACGATAAGACATACACCAAGGAA
KF517285.1      ACCGACAATGGTCTAACTCCACCCAGATTAAATAAAGACGATAAGACATACACCAAGGAA
                ************************************************************

NORM            GAATTAATGGCGCCGGATGATTACGCATGTACAGATCATTTTGATAGGGTTGAAGTTAAG
KF517285.1      GAATTAATGGCGCCGGATGATTACGCATGTACAGATCATTTTGATAGGGTTGAAGTTAAG
                ************************************************************

NORM            AAATCTTATGAGTTAATTCAAGACCATTTCGGTTGCGAATACAAGTTGTACTGTAAAATA
KF517285.1      AAATCTTATGAGTTAATTCAAGACCATTTCGGTTGCGAATACAAGTTGTACTGTAAAATA
                ************************************************************

NORM            ACGCCTCATAACGTTAGATGTTACATCACCAATTTCCCTCAATGTCAAACACCCGCATAC
KF517285.1      ACGCCTCATAACGTTAGATGTTACATCACCAATTTCCCTCAATGTCAAACACCCGCATAC
                ************************************************************

NORM            ATATCTGGCACCATAGGATCCGACACAATACCCAACACTGCACTGACCCCAAAGAGCCTG
KF517285.1      ATATCTGGCACCATAGGATCCGACACAATACCCAACACTGCACTGACCCCAGAGAGCCTG
                ************************************************ ********

NORM            TCAGTCATGTTTATAAAAGGGGGATTGGTCTCTACAACATCGTTAGATTTGTCAATATGG
KF517285.1      TCAGTCATGTTTATAAAAGGGGGATTGGTCTCTACAACATCGTTAGATTTGTCAATATGG
                ************************************************************

NORM            ACGATAAAAGGAATTAAATTAGCACAATTCACAACAGCAGCAGATTTACCGGATGCCTGT
KF517285.1      ACGATAAAAGGAATTAAATTAGCACAATTCACAACAGCAGCAGATTTACCGGATGCCTGT
                ************************************************************

NORM            GAATTGGCGGCGAATAACATACAAGTCACGCATAACATGGACTTTACATCCGCCGGGAAA
KF517285.1      GAATTGGCGGCGAATAACATACAAGTCACGCATAACATGGACTTTACATCCGCCGGGAAA
                ************************************************************

NORM            ACCGTCACTTTCGCCTGCATAAACAAATTACCGCTGGACAACACATGCGATATATCTGCA
KF517285.1      ACCGTCACTTTCGCCTGCATAAACAAATTACCGCTGGACAACACATGCGATATATCTGCA
                ************************************************************

NORM            GGACATTCAAAAGACACTCAATACAAATTGGAAATAAGTAATGATGGAAGTAATTGGGTG
KF517285.1      GGACATTCAAAAGACACTCAATACAAATTGGAAATAAGTAATGATGGAAGTAATTGGGTG
                ************************************************************

NORM            GCATTAGCAGAGTCTACATTGGCCATTGATGGTAGTGGTGTCAAAACATTAACATCGACA
KF517285.1      GCATTAGCAGAGTCTACATTGGCCATTGATGGTAGTGGTGTCAAAACATTAACATCGACA
                ************************************************************

NORM            TTCAGTAAAGAGGGAGGGATATTTGCGGAAGGAGACGGTGTATTTTCGTTTTTATTTTAC
KF517285.1      TTCAGTAAAGAGGGAGGGATATTTGCGGAAGGAGACGGTGTATTTTCGTTTTTATTTTAC
                ************************************************************

NORM            TCATTGAATGATGATGCAATTAGAACCATGTATACTGACAGGAGCAACATACAGGCCAGG
KF517285.1      TCATTGAATGATGATGCAATTAGAACCATGTATACTGACAGGAGCAACATACAGGCCAGG
                ************************************************************

NORM            TGTGTAAAGATGTTCGATTCCTCATCTTCAACATCCACAATAAAGGCTGTAGATTACATA
KF517285.1      TGTGTAAAGATGTTCGATTCCTCATCTTCAACATCCACAATAAAGGCTGTAGATTACATA
                ************************************************************

NORM            AGCTACGACACATATAGGAAATCCCTAGTTCCTGAAGAACCCACAGTAACAACTACTACT
KF517285.1      AGCTACGACACATATAGGAAATCCCTAGTTCCTGAAGAACCCACAGTAACAACTACTACT
                ************************************************************

NORM            GAATCACCACCTCCTCCAACAACCACTACCAGACAGATACATTCCAAAGAAGATTTCGAC
KF517285.1      GAATCACCACCTCCTCCAACAACCACTACCAGACAGATACATTCCAAAGAAGATTTCGAC
                ************************************************************

NORM            AGGGTTAAAAAAGAACTCGGTGAAAAACTTTATCATGTTTTATTCTTTATGGGTGTTTTA
KF517285.1      AGGGTTAAAAAAGAACTCGGTGAAAAACTTTATCATGTTTTATTCTTTATGGGTGTTTTA
                ************************************************************

NORM            ACAGTATCTGTTGCCGGCGGTGTGATTATACTATCATTTATTGGCTGCCTGATAATGCGC
KF517285.1      ACAGTATCTGTTGCCGGCGGTGTGATTATACTATCATTTATTGGCTGCCTGATAATGCGC
                ************************************************************

NORM            AAGATGGAAGATGCACCACAAAAGACAAAATATAGTGTATAG
KF517285.1      AAGATGGAAGATGCACCACAAAAGACAAAATATAGTGTATAG
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NORM            TCACAAAGGAAGTAAACTACCATTCATCATCTTTTGCCGCAACATCACAACAATTATAAC
KF517264.1      TCACAAAGGAAGTAAACTACCATTCATCATCTTTTGCCGCAACATCACAACAATTATAAC
                ************************************************************

NORM            AAGTAGTAAAATCCCACTGCCACCCCGGCGCCAATTCCTATAATCGTCCAATGATATCCC
KF517264.1      AAGTAGTAAAATCCCACTGCCACCCCGGCGCCAATTCCTATAATCGTCCAATGATATCCC
                ************************************************************

NORM            GGTGCCAGATCAGGAATGGGTTTAAGTATTGCGCCTGTTTTGTTGAGTTCATCTCTAAAC
KF517264.1      GGTGCCAGATCAGGAATGGGTTTAAGTATTGCGCCTGTTTTGTTGAGTTCATCTCTAAAC
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                ************************************************************
NORM            TTCACAAGTTCTTCTAGTTTATAATCCTCCATGGTTTTTGTTACAGGTTTCTTATTGTTT
KF517264.1      TTCACAAGTTCTTCTAGTTTATAATCCTCCATGGTTTTTGTTACAGGTTTCTTATTGTTT
                ************************************************************

NORM            TCTTGGGAAGGTGGTTTTGTTGTTACTGGTACTACCACTGGTTTTTGTGTTGTTGGGGGA
KF517264.1      TCTTGGGAAGGTGGTTTTGTTGTTACTGGTACTACCACTGGTTTTTGTGTTGTTGGGGGA
                ************************************************************

NORM            GGTGGAACTGTCACCCTTGTGGTAGTGGCTTCTGTGGTGGTAGTTGTTGTAGTTGGTAAA
KF517264.1      GGTGGAACTGTCACCCTTGTGGTAGTGGCTTCTGTGGTGGTAGTTGTTGTAGTTGGTAAA
                ************************************************************

NORM            GGAAGTGTTATGGAATTTTTACTTTCAACTCCAGCAAAATAACATGTATAGGAACCAGGT
KF517264.1      GGAAGTGTTATGGAATTTTTACTTTCAACTCCAGCAAAATAACATGTATAGGAACCAGGT
                ************************************************************

NORM            GTGTCATCGACTTCAAATTCTATACTAGAAAATGTTTTACCCGTCTGAGGATCAACAGAT
KF517264.1      GTGTCATCGACTTCAAATTCTATACTAGAAAATGTTTTACCCGTCTGAGGATCAACAGAT
                ************************************************************

NORM            CCACATCTGTTTGCTCTCTGTTTGGTACCGCAACTGTTTTGTGTGGTGATTGTATTAAAC
KF517264.1      CCACATCTGTTTGCTCTCTGTTTGGTACCGCAACTGTTTTGTGTGGTGATTGTATTAAAC
                ************************************************************

NORM            ACCTCCCCAAATGGCACCGTTCTAAAATCCTCGTGTCTTTTCGATCCTATTTTTATTATA
KF517264.1      ACCTCCCCAAATGGCACCGTTCTAAAATCCTCGTGTCTTTTCGATCCTATTTTTATTATA
                ************************************************************

NORM            TCCTTCACGATGTCGTAAAAGGTGTTTCTATATTCAGATTTCTTTCTGTTTCTATTGGTA
KF517264.1      TCCTTCACGATGTCGTAAAAGGTGTTTCTATATTCAGATTTCTTTCTGTTTCTATTGGTA
                ************************************************************

NORM            CATGTTGAATCCAACATAATATTATCTTCGGCCAGTGTGGTACTGTCGTAATTTGTTTCC
KF517264.1      CATGTTGAATCCAACATAATATTATCTTCGGCCAGTGTGGTACTGTCGTAATTTCTTTCC
                ************************************************** *****

NORM            GGGTATAATTTAATTTCCAAACCATCCAGGGTGGGATTTACATATGTTGGTTTCTTGTCG
KF517264.1      GGGTATAATTTAATTTCCAAACCATCCAGGGTGGGATTTACATATGTTGGTTTCTTGTCG
                ************************************************************

NORM            TAAAAGATGTAGAAATATTTACTACCATGATTGGCTTGTGGAGCACCTCTTTCCGGAACC
KF517264.1      TAAAAGATGTAGAAATATTTACTACCATGATTGGCTTGTGGAGCACCTCTTTCCGGAACC
                ************************************************************

NORM            CATAGGTAGATTTGTTGAATATCGTCGTGAACTGTTACCTTTCCCGCTACAACTCTAAGC
KF517264.1      CATAGGTAGATTTGTTGAATATCGTCGTGAACTGTTACCTTTCCCGCTACAACTCTAAGC
                ************************************************************

NORM            GTACAACCCTCTCCTGAATCTGCCATGTTTAAGTAATTGGTGTTGAGTTGATTAATCTGC
KF517264.1      GTACAACCCTCTCCTGAATCTGCCATGTTTAAGTAATTGGTGTTGAGTTGATTAATCTGC
                ************************************************************

NORM            CTTTGACCGAGAAATGCAATTTCCTGCCCTTGAAAATTTGTTAAATCGATTTGCGGTCCT
KF517264.1      CTTTGACCGAGAAATGCAATTTCCTGCCCTTGAAAATTTGTTAAATCGATTTGCGGTCCT
                ************************************************************

NORM            TTAACAATCCTTGCCTTTTTAAACATGGACATATATTCTATCATTTTTTTCAAAAAGGTA
KF517264.1      TTAACAATCCTTGCCTTTTTAAACATGGACATATATTCTATCATTTTTTTCAAAAAGGTA
                ************************************************************

NORM            AACGTCTGAGAAGGTGCCAAAGGGAAATGCACTGATTTATAATCATATGGAACGTACGTT
KF517264.1      AACGTCTGAGAAGGTGCCAAAGGGAAATGCACTGATTTATAATCATATGGAACGTACGTT
                ************************************************************

NORM            ACCATATTCGTATCCAAATCATATTCTATTCCCTCTGCTGGGATAACAGGTGAATCCACG
KF517264.1      ACCATATTCGTATCCAAATCATATTCTATTCCCTCTGCTGGGATAACAGGTGAATCCACG
                ************************************************************

NORM            TATGGCAATTTTAATCCAAATTTAAAATGTCGATTTTCCGGAACTGTATTGACGTTATAA
KF517264.1      TATGGCAATTTTAATCCAAATTTAAAATGTCGATTTTCCGGAACTGTATTGACGTTAAAA
                ************************************************************ **

NORM            TTTTCATTCGTTCCAATCACTATGGAATTCATACACGACAATGCAACCTTTTCCATAATT
KF517264.1      TTTTCATTCGTTCCAATCACTATGGAATTCATACACGACAATGCAACCTTTTCCATAATT
                ************************************************************

NORM            GGAGTTTTCGAAATTGGTTGGTTACAGTCAACCAACTCTCTGGTGACAGCCTCGCTCCTT
KF517264.1      GGAGTTTTCGAAATTGGTTGGTTACAGTCAACCAACTCTCTGGTGACAGCCTCGCTCCTT
                ************************************************************

NORM            CTATAACGACCATATACATCATCTTCCAATATTTTGACCCCGTCTCGTTTGATCCATGTC
KF517264.1      CTATAACGACCATATACATCATCTTCCAATATTTTGACCCCGTCTCGTTTGATCCATGTC
                ************************************************************

NORM            AATGGTCACATTTTATATTCTGTCAATCTATCATCGGGAAGGTCGTATGTGTGTATATCT
KF517264.1      AATGGTCTCATTTTATATTCTGTCAATCTATCATCGGGAAGGTCGTATGTGTGTATATCT
                ******* ****************************************************

NORM            CGTAAATCTAAACCCTCAGAACCACATCCTAAAATAACTTTTCCTGGAGCGCTTGAAACA
KF517264.1      CGTAAATCTAAACCCTCAGAACCACATCCTAAAATAACTTTTCCTGGAGCGCTTGAAACA
                ************************************************************

NORM            ACCTTGATCACTGTTGTATTTTCACGAGATTCTATAGACCCAATTCGTTTAATAGAGTCC
KF517264.1      ACCTTGATCACTGTTGTATTTTCACGAGATTCTATAGACCCAATTCGTTTAATAGAGTCC
                ************************************************************

NORM            ATACTATCCTTTGCGCTATAAGTGGATATTCTTCCATCCACATTCTGTGTCCATGTAATC
KF517264.1      ATACTATCCTTTGCGCTATAAGTGGATATTCTTCCATCCACATTCTGTGTCCATGTAATC
                ************************************************************

NORM            CAATATGAATTAAAGGCGTCCACGTCATTATTGATGTTGGGTATCTCAATAAACATTCTC
KF517264.1      CAATATGAATTAAAGGCGTCCACGTCATTATTGATGTTGGGTATCTCAATAAACATTCTC
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                  ************************************************************
NORM              CACATATCACCATTCTTTGAGCAGGCGTGCCATATACCAGGAGGTTCCATGGCACAACCT
KF517264.1        CACATATCACCATTCTTTGAGCAGGCGTGCCATATACCAGGAGGTTCCATGGCACAACCT
                  ************************************************************
NORM              GAATTGGCTGTTATATTAAAACTAAAGGGTGGGTAGTCATCATTGCTAAATTGATGAACT
KF517264.1        GAATTGGCTGTTATATTAAAACTAAAGGGTGGGTAGTCATCATTGCTAAATTGATGAACT
                  ************************************************************
NORM              ATATCTTCATGCTGGGACTGGGTAATTAAAAATTGTTTTATCACGGCGTTGCGTAATTAA
KF517264.1        ATATCTTCATGCTGGGACTGGGTAATTAAAAATTGTTTTATCACGGCGTTGCGTAATTAA
                  ************************************************************
NORM              AACAGAATCGGAATTGTGTAAAAACAACAATGCTATAAATAAACCTGTTATATACATTCT
KF517264.1        AACAGAATCGGAATTGTGTAAAAACAACAATGCTATAAATAAACCTGTTATATACATTCT
                  ************************************************************
NORM              TGTCAT
KF517264.1        TGTCAT
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NORM              ATGGCAACAGACCAACAAGACCTCGACATTATCAGCAGCACAGCTGAACTCAGAGGGGCA
KF517324.1        ATGGCAACAGACCAACAAGACCTCGACATTATCAGCAGCACAGCTGAACTCAGAGGGGCA
                  ************************************************************
NORM              TGTGATTTCTGGGAAACCAGATCAGGTGGAGTCACAACAATAACGATTACTAGAATTAAC
KF517324.1        TGTGATTTCTGGGAAACCAGATCAGGTGGAGTCACAACAATAACGATTACTAGAATTAAC
                  ************************************************************
NORM              AGGGATGCTATCGTGTTATTGGCTGGTGTATGCCCCGGAGAATCATTTTCCGTATCGTAC
KF517324.1        AGGGATGCTATCGTGTTATTGGCTGGTGTATGCCCCGGAGAATCATTTTCCGTATCGTAC
                  ************************************************************
NORM              AACAAGGAAAAGATTCTGGTCAATTCTTATCCCTTTAACATTAATAACGTGGATGTCGTG
KF517324.1        AACAAGGAAAAGATTCTGGTCAATTCTTATCCCTTTAACATTAATAACGTGGATGTCGTG
                  ************************************************************
NORM              GGTGGTACTACAGATATAAATGATTTCAATAGCAAGATGAAGTCACTTTACCTCCCCGTC
KF517324.1        GGTGGTACTACAGATATAAATGATTTCAATAGCAAGATGAAGTCACTTTACCTCCCCGTC
                  ************************************************************
NORM              AATGGTATGACCGTGTTAATGCTTACAGAGGGAAGAATTAACAACCCAGAAATTGCGGTG
KF517324.1        AATGGTATGACCGTGTTAATGCTTACAGAGGGAAGAATTAACAACCCAGAAATTGCGGTG
                  ************************************************************
NORM              GTCACAGAAGATGGGAATTTAGAGGTTGTAGGAAGCAAGAAAAAGACATTGGTCAAATTG
KF517324.1        GTCACAGAAGATGGGAATTTAGAGGTTGTAGGAAGCAAGAAAAAGACATTGGTCAAATTG
                  ************************************************************
NORM              TTATTGTTATTTTTATCACTTATGGTGGTGATTGTAGGGGTGTGGGTGGGAAGTATTTTT
KF517324.1        TTATTGTTATTTTTATCACTTATGGTGGTGATTGTAGGGGTGTGG--TGGAAGTATTTTT
                  ********************************************   ************
NORM              CCACGAGTGAATTATCAGCCAGTGCCTTATTTGACACTGTTGGACAGAGTGTAAAATCGA
KF517324.1        CCACGAGTGAATTATCAGCCAGTGCCTTATTTGACACTGTTGGACAGAGTGTAAAATCGA
                  ************************************************************
NORM              AAGGGAATTATGAAGACCTCTTTAAGTAA
KF517324.1        AAGGGAATTATGAAGACCTCTTTAAGTAA
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ORF 80

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NORM              ATGGGTGATAATACAACAGTAGCTCCTGGTACAAACCAGACTCTTGTTGAAGAGGATTTG
KF517317.1        ATGGGTGATAATACAACAGTAGCTCCTGGTACAAACCAGACTCTTGTTGAAGAGGATTTG
                  ************************************************************
NORM              GGTGCACAGATTACCCATACACTCATGGTTCAAATCATGTCAAAATTAAATGAAATGCTA
KF517317.1        GGTGCACAGATTACCCATACACTCATGGTTCAAATCATGTCAAAATTAAATGAAATGCTA
                  ************************************************************
NORM              ACAGAATACCAACCACAGATTATTGGGATTGGTGCAACAGTATTGGCAATTTTTGTTATA
KF517317.1        ACAGAATACCAACCACAGATTATTGGGATTGGTGCAACAGTATTGGCAATTTTTGTTATA
                  ************************************************************
NORM              ATGTTTATTTCATTACTGATAATCCTGGGATGCAACTGTATACGACCATTCAACTTCAAG
KF517317.1        ATGTTTATTTCATTACTGATAATCCTGGGATGCAACTGTATACGACCATACAACTTCAAG
                  ********************************************** **********
NORM              AACCTGAAACGATACATCACCGGCAAGGCATCGAAGTCAGTTGAATATCAACCATTGAAA
KF517317.1        AACCTGAAACGATACATCACCGGCAAGGCATCGAAGTCAGTTGAATATCAACCATTGAAA
                  ************************************************************
NORM              ATGTCAGCAGTAAACATGGGAATGGATGAAGACGATGAATTCCTTGTCTAA
KF517317.1        ATGTCAGCAGTAAACATGGGAATGGATGAAGACGATGAATTCCTTGTCTAA
                  ***************************************************
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KF517256.1      ATGATCATTATGAAATCCATAATATTATTACTCGCTTGGTTTTTAACAAAAACACAGGCG
KF517258.1      ATGATCATTATGAAATCCATAATATTATTACTCGCTTGGTTTTTAACAAAAACACAGGCG
NORM            ATGATCATTATGAAATCCATAATATTATTACTCGCTTGGTTTTTAACAAAAACACAGGCG
                ************************************************************

KF517256.1      AATATGTTGACCGAATCTTTGTACTTGTCAGAATATGAGGGGAGTGTTGTGCTAAACATC
KF517258.1      AATATGTTGACCGAATCTTTGTACTTGTCAGAATATGAGGGGAGTGTTGTGCTAAACATC
NORM            AATATGTTGACCGAATCTTTGTACTTGTCAGAATATGAGGGGAGTGTTGTGCTAAACATC
                ************************************************************

KF517256.1      ATAGACAAAAATTTAAACGGGATATCCACCCTGTCAATATTTAACGATTCTACTAAATTA
KF517258.1      ATAGACAAAAATTTAAACGGGATATCCACCCTGTCAATATTTAACGATTCTACTAAATTA
NORM            ATAGACAAAAATTTAAACGGGATATCCACCCTGTCAATATTTAACGATTCTACTAAATTA
                ************************************************************

KF517256.1      CAAGAAGTAAGATATGTTGCCAGTGTATGCAGCCTCAGATCTGGATCGTTCAATATCACT
KF517258.1      CAAGAAGTAAGATATGTTGCCAGTGTATGCAGCCTCAGATCTGGATCGTTCAATATCACT
NORM            CAAGAAGTAAGATATGTTGCCAGTGTATGCAGCCTCAGATCTGGATCGTTCAATATCACT
                ************************************************************

KF517256.1      TGCAATGTAATAACCTACGGCACTTATCATGTCAGGATGTTTTTATCAGGTCTTAACATG
KF517258.1      TGCAATGTAATAACCTACGACACTTATCATGTCAGGATGTTTTTATCAGGTCTTAACATG
NORM            TGCAATGTAATAACCTACGGCACTTATCATGTCAGGATGTTTTTATCAGGTCTTAACATG
                ******************* ****************************************

KF517256.1      TCCGCATTTGATTTATACAGACTTCGATATGTGTACGTTGGTCTTAGAGACGCCATAAAT
KF517258.1      TCCGCATTTGATTTATACAGACTTCGATATGTGTACGTTGGTCTTAGAGACGCCATAAAT
NORM            TCCGCATTTGATTTATACAGACTTCGATATGTGTACGTTGGTCTTAGAGACGCCATAAAT
                ************************************************************

KF517256.1      TACAACCCAAAATATGCAGAGGCGGTAATGGCACCGTTTGCTTTAATTGGCAATAATAAT
KF517258.1      TACAACCCAAAATATGCAGAGGCGGTAATGGCACCGTTTGCTTTAATTGGCAATAATAAT
NORM            TACAACCCAAAATATGCAGAGGCGGTAATGGCACCGTTTGCTTTAATTGGCAATAATAAT
                ************************************************************

KF517256.1      ATAGTTACAATTAAACTTATAAAAGACGGTGATAATATCACCGTCGGTTGTGGGTTTGGA
KF517258.1      ATAGTTACAATTAAACTTATAAAAGACGGTGATAATATCACCGTCGGTTGTGGGTTTGGA
NORM            ATAGTTACAATTAAACTTATAAAAGACGGTGATAATATCACCGTCGGTTGTGGGTTTGGA
                ************************************************************

KF517256.1      AATGTAGATTTGAGCACTGTAAACACCCATGCCAGCAAAATTGGCAGAAATATAAACCCT
KF517258.1      AATGTAGATTTGAGCACTGTAAACACCCATGCCAGCAAAATTGGCAGAAATATAAACCCT
NORM            AATGTAGATTTGAGCACTGTAAACACCCATGCCAGCAAAATTGGCAGAAATATAAACCCT
                ************************************************************

KF517256.1      AGATTCATGGTCGGTGTTTATACAAACGATAGCAATAAGTTAATCGAGGATGATATATAC
KF517258.1      AGATTCATGGTCGGTGTTTATACAAACGATAGCAATAAGTTAATCGAGGATGATATATAC
NORM            AGATTCATGGTCGGTGTTTATACAAACGATAGCAATAAGTTAATCGAGGATGATATATAC
                ************************************************************

KF517256.1      AGCCGTTACACAGATTCAGAATCTGCCGGTGTTATGAGAAAATGTAATTTAAACGAGGTT
KF517258.1      AGCCGTTACACAGATTCAGAATCTGCCGGTGTTATGAGAAAATGTAATTTAAACGAGGTT
NORM            AGCCGTTACACAGATTCAGAATCTGCCGGTGTTATGAGAAAATGTAATTTAAACGAGGTT
                ************************************************************

KF517256.1      AAAACCACTCCCCAGGAAGATTGCATTCAGCCCTTTTGCACCAAAGGAACAGTGTATGGA
KF517258.1      AAAACCACTCCCCAGGAAGATTGCATTCAGCCCTTTTGCACCAAAGGAACAGTGTATGGA
NORM            AAAACCACTCCCCAGGAAGATTGCATTCAACCCTTTTGCACCAAAGGAACAGTGTATGGA
                ***************************** ****************************

KF517256.1      AATAACCTCGTTTATGGAAGTAGATTACGATGTTTCAGTAGGACCAGGTGTTCACAGAGA
KF517258.1      AATAACCTCGTTTATGGAAGTAGATTACGATGTTTCAGTAGGACCAGGTGTTCACAGAGA
NORM            AATAACCTCGTTTATGGAAGTAGATTACGATGTTTCAGTAGGACCAGGTGTTCACAGAGA
                ************************************************************

KF517256.1      AGTAGAACCGTTCCTCAATCAGTCCCTTGGTATATACCATCTGGATTTACAGAAAAACAG
KF517258.1      AGTAGAACCGTTCCTCAATCAGTCCCTTGGTATATACCATCTGGATTTACAGAAAAACAG
NORM            AGTAGAACCGTTCCTCAATCAGTCCCTTGGTATATACCATCTGGATTTACAGAAAAACAG
                ************************************************************

KF517256.1      TTTATGTACCTGGATAATAGACTGGGATATCTTTTGGGATTAGACCTTACCACGGCTATT
KF517258.1      TTTATGTACCTGGATAATAGACTGGGATATCTTTTGGGATTAGACCTTACCACGGCTATT
NORM            TTTATGTACCTGGATAATAGACTGGGATATCTTTTGGGATTAGACCTTACCACGGCTATT
                ************************************************************

KF517256.1      TTTAAATATACCCCAATTGTTGTCGGACATATAGTAAGTGAATACCTGACGGGAATCATG
KF517258.1      TTTAAATATACCCCAATTGTTGTCGGACATATAGTAAGTGAATACCTGACGGGAATCATG
NORM            TTTAAATATACCCCAATTGTTGTCGGACATATAGTAAGTGAATACCTGACGGGAATCATG
                ************************************************************

KF517256.1      AACTATGAGCGTCTCAGTGTCAGGAAAGGACCGTATATAGACATGCGAGGTATTATAGGT
KF517258.1      AACTATGAGCGTCTCAGTGTCAGGAAAGGACCGTATATAGACATGCGAGGTATTATAGGT
NORM            AACTATGAGCGTCTCAGTGTCAGGAAAGGACCGTATATAGACATGCGAGGTATTATAGGT
                ************************************************************

KF517256.1      GGAGAAATCAAAATGATATTGATAAGAAACTACAGAAAGATGTTGGACATGAGTGGATTT
KF517258.1      GGAGAAATCAAAATGATATTGATAAGAAACTACAGAAAGATGTTGGACATGAGTGGATTT
NORM            GGAGAAATCAAAATGATATTGATAAGAAACTACAGAAAGATGTTGGACATGAGTGGATTT
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KF517256.1      ACACCTTTGCCGGCGAATGGATGTTATGTCACAGTGATAAAATTCATCGGGGATAAACGC
KF517258.1      ACACCTTTGCCGGCGAATGGATGTTATGTCACAGTGATAAAATTCATCGGGGATAAACGC
NORM            ACACCTTTGCCGGCGAATGGATGTTATGTCACAGTGATAAAATTCATCGGGGATAAACGC
                ************************************************************

KF517256.1      GTTTTTAATCGAGTTTGGATGCCTAGTAAAAACACCAATGAGGGAGAAGAACATGTTTTC
KF517258.1      GGTTTTAATCGAGTTTGGACGCCTAGTAAAAACACCAATGAGGGAGAAGAACATGTTTTC
NORM            GGTTTTAATCGAGTTTGGATGCCTAGTAAAAACACCAATGAGGGAGAAGAACATGTTTTC
                * ***************** ****************************************

KF517256.1      GTCTTTCATCAAAAAAGGTTAAGCAATATAAGTGATTATACTTTAAGAATATTCCCCGAT
KF517258.1      GTCTTTCATCAAAAAAGGTTAAGCAATATAAGTGATTATACTTTAAGAATATTCCCCGAT
NORM            ATCTTTCATCAAAAAAGGTTAAGCAATATAAGTGATTATACTTTAAGAATATTCCCCGAT
                 ************************************************************

KF517256.1      AGCGGTATGGACACAGAGGGAAGTAAATATACAATGAACACCATAACAGATGTGGGTTGT
KF517258.1      AGCGGTATGGACACAGAGGGAAGTAAATATACAATGAACACCATAACAGATGTGGGTTGT
NORM            AGCGGTATGGACACAGAGGGAAGTAAATATACAATGAACACCATAACAGATGTGGGTTGT
                ************************************************************

KF517256.1      AGTCGTGAAACCCACCACAAATCGGTTTATCCCGCAACAATCAAAAAGGCAATAGAAAGG
KF517258.1      AGTCGTGAAACCCACCACAAATCGGTTTATCCCGCAACAATCAAAAAGGCAATAGAAAGG
NORM            AGTCGTGAAACCCACCACAAATCGGTTTATCCCGCAACAATCAAAAAGGCAATAGAAAGG
                ************************************************************

KF517256.1      TTCTGCGTCGACCAACCTAATATATCATGTGAATATGTTAAGGATATTGACAGGGTTGAT
KF517258.1      TTCTGCGTCGACCAACCTAATATATCATGTGAATATGTTAAGGATATTGACAGGGTTGAT
NORM            TTCTGCGTCGACCAACCTAATATATCATGTGAATATGTTAAGGATATTGACAGGGTTGAT
                ************************************************************

KF517256.1      ATTAACCCTTGCGGATGTAAACAAAGAGCCAACAGATGTGGTGAGCGGTATTCCAACAAC
KF517258.1      ATTAACCCTTGCGGATGTAAACAAAGAGCCAACAGATGTGGTGAGCGGTATTCCAACAAC
NORM            ATTAACCCTTGCGGATGTAAACAAAGAGCCAACAGATGTGGTGAGCGGTATTCCAACAAC
                ************************************************************

KF517256.1      ACACTTAAAGCAACTATAGAATTTGAAGTGCCAAAGATTTACGATACACCTTACACGTGT
KF517258.1      ACACTTAAAGCAACTATAGAATTTGAAGTGCCAAAGATTTACGATACACCTTACACGTGT
NORM            ACACTTAAAGCAACTATAGAATTTGAAGTGCCAAAGATTTACGATACACCTTACACGTGT
                ************************************************************

KF517256.1      GAATTCTTGGGATATAAAAGTGTGAATTCATTAACATTCGATTCACCACCACCGCCACCA
KF517258.1      GAATTCTTGGGATATAAAAGTGTGAATTCATTAACATTCGATTCACCACCACCGCCACCA
NORM            GAATTCTTGGGATATAAAAGTGTGAATTCATTAACATTCGATTCACCACCACCGCCACCA
                ************************************************************

KF517256.1      ACTACCACTCAGGCGCCTCCTCCACCACCCACCACCACTCAAGCTCCTCCACCCCCGCCA
KF517258.1      ACTACCACTCAGGCGCCTCCTCCACCACCCACCACCACTCAAGCTCCTCCACCCCCGCCA
NORM            ACTACCACTCAGGCGCCTCCTCCACCACCCACCACCACTCAAGCTCCTCCACCCCCGCCA
                ************************************************************

KF517256.1      ACCACCACACAAGCTCCTCCTCCACCTATCGTTATTAATACCACAGCAGCACCTTTGGCG
KF517258.1      ACCACCACACAAGCTCCTCCTCCACCTATCGTTATTAATACCACAGCAGCACCTTTGGCG
NORM            ACCACCACACAAGCTCCTCCTCCACCTATCGTTATTAATACCACAGCAGCACCTTTGGCG
                ************************************************************

KF517256.1      CCCATTACCAATGCCACATTGCCACCAAGTGATGTGATCACACCGGAGGCTGTGAATTTA
KF517258.1      CCCATTACCAATGCCACATTGCCACCAAGTGATGTGATCACACCGGAGGCTGTGAATTTA
NORM            CCCATTACCAATGCCACATTGCCACCAAGTGATGTGATCACACCGGAGGCTGTGAATTTA
                ************************************************************

KF517256.1      ACAGATGATACCCCTGTTGTAAATGAACCGGTAAATTCTACATTTATCAATGATACGGAT
KF517258.1      ACAGATGATACCCCTGTTGTAAATGAACCGGTAAATTCTACATTTATCAATGATACGGAT
NORM            ACAGATGATACCCCTGTTGTAAATGAACCGGTAAATTCTACATTTATCAATGATACGGAT
                ************************************************************

KF517256.1      GTATTAGATGATTCTCCCACCACCTCTGCTCCACAAGCGCCTGGTATAGTTGGTATAATT
KF517258.1      GTATTAGATGATTCTCCCACCACCTCTGCTCCACAAGCGCCTGGTATAGTTGGTATAATT
NORM            GTATTAGATGATTCTCCCACCACCTCTGCTCCACAAGCGCCTGGTATAGTTGGTATAATT
                ************************************************************

KF517256.1      GTAAATAAGATTACAACAACACCTGCACCATCCATCGGTAGGGTGCCTATCCCACCACCA
KF517258.1      GTAAATAAGATTACAACAACACCTGCACCATCCATCGGTAGGGTGCCTATCCCACCACCA
NORM            GTAAATAAGATTACAACAACACCTGCACCATCCATCGGTAGGGTGCCTATCCCACCACCA
                ************************************************************

KF517256.1      GATGTACCAGTTGAACCACCCAGATCTATCCCTACAACCAACGCACCTTCACCCGAAGAG
KF517258.1      GATGTACCAGTTGAACCACCCAGATCTATCCCTACAACCAACGCACCTTCACCCGAAGAG
NORM            GATGTACCAGTTGAACCACCCAGATCTATCCCTACAACCAACGCACCTTCACCCGAAGAG
                ************************************************************

KF517256.1      GATACAGTGGTTTTATCTAAATCTGACATTATGCGACGGTTTTTGATAAGGTTAAAGACT
KF517258.1      GATACAGTGGTTTTATCTAAATCTGACATTATGCGACGGTTTTTGATAAGGTTAAAGACT
NORM            GATACAGTGGTTTTATCTAAATCTGACATTATGCGACGGTTTTTGATAAGGTTAAAGACT
                ************************************************************

KF517256.1      AGAGATGGAGAAACCGTCGATATTTATACATGGCCAGAACTTAACCTTGCGCCTTTTAAA
KF517258.1      AGAGATGGAGAAACCGTCGATATTTATACATGGCCAGAACTTAACCTTGCGCCTTTTAAA
NORM            AGAGATGGAGAAACCGTCGATATTTATACATGGCCAGAACTTAACCTTGCGCCTTTTAAA
                ************************************************************

KF517256.1      ACTTTAAGCTATGCCGGAATTGGTGTCGTGTCATTTGCTTTGTTATTCACTATTCTAGTT
KF517258.1      ACTTTAAGCTATGCCGGAATTGGTGTCGTGTCATTTGCTTTGTTATTCACTATTCTAGTT
NORM            ACTTTAAGCTATGCCGGAATTGGTGTCGTGTCATTTGCTTTGTTATTCACTATTCTAGTT
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KF517256.1      GTCTGCTTAATAAAATTCTCAATATAG
KF517258.1      GTCTGCTTAATAAAATTCTCAATATAG
NORM            GTCTGCTTAATAAAATTCTCAATATAG
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NORM            ATGTTGCGATCAAAAAGAACAAAGGTTGTTTACAAAACAGCCGATGTAATATTACCACCA
KF517305.1      ATGTTGCGATCAAAAAGAACAAAGGTTGTTTACAAAACAGCCGATGTAATATTACCACCA
                ************************************************************

NORM            GTAGAAGAAAATATCAATAGCGAGAATAAAAATGAAGGGGAGGTGAAGGTGACAACCTTT
KF517305.1      GTAGAAGAAAATATCAATAGCGAGAATAAAAATGAAGGGGAGGTGAAGGTGACAACCTTT
                ************************************************************

NORM            GCTGATGAACCCAAGATAGAACAAGAGGAACCTCAACAAAAACCAGAGGTGGTTGATGTA
KF517305.1      GCTGATGAACCCAAGATAGAACAAGAGGAACCTCAACAAAAACCAGAGGTGGTTGATGTA
                ************************************************************

NORM            TATAGTAATGAAACGGATAAGAATGAAGAAGAGGTGTCTATAATAACATCTGAAGATGAA
KF517305.1      TATAGTAATGAAACGGATAAGAATGAAGAAGAGGTGTCTATAATAACATCTGAAGATGAA
                ************************************************************

NORM            GAGGAAGACGAAAAGGGCATGTTGTTTAAGAGACCGGGTAAAAAAAACACAATTATGCA
KF517305.1      GAGGAAGACGAAAAGGGCATGTTGTTTAAGAGACCGGGTGGAAAAAACACAATTATGCA
                ************************************  ******************

NORM            CCCAGTAAATATGTAGGCGAAGAATTTGATCTAGACGCCCTTAAAGAACATAGAAAAATG
KF517305.1      CCCAGTAAATATGTAGGCGAAGAATTTGATCTAGACGCCCTTAAAGAACATAGAAAAATG
                ************************************************************

NORM            GTCAAGAGATGGATAATTGGCATAAGTGTCAGATTTGGATTTTGGTTCGCGTTGTTGATT
KF517305.1      GTCAAGAGATGGATAATTGGCATAAGTGTCAGATTTGGATTTTGGTTCGCGTTGTTGATT
                ************************************************************

NORM            CCTGTTGCTATTTTGTTGAGACCATATACAATAGAATGTGAACCGATCAACACCTTTTCG
KF517305.1      CCTGTTGCTGTTTTGTTGAGACCATATACAATAGAATGTGAACCGATCAACACCTTTTCG
                ********* ************************************************

NORM            GAATTCTCATTGTGCGTGATATTGTTTTTGTTGCTACAGGCGGGTATAGACCTTGGCTTA
KF517305.1      GAATTCTCATTGTGCGTGATATTGTTTTTGTTGCTACAGGCGGGTATAGACCTTGGCTTA
                ************************************************************

NORM            GCTATTTTTTCATACAGAAAGGTAGGGAAGTTCTTGGAATCACCAGCGGTGGATGAGATT
KF517305.1      GCTATTTTTTCATACAGAAAGGTAGGGAAGTTCTTGGAATCACCAGCGGTGGATGAGATT
                ************************************************************

NORM            AACATTCTTATGGCCATGAAACCCACAGGTGGCGTCATGGGTAATCCACATGCAAATACG
KF517305.1      AACATTCTTATGGCCATGAAACCCACAGGTGGCGTCATGGGTAATCCACATGCAAATACG
                ************************************************************

NORM            GAGGCATTGGCAGCAAGTGTAAAGATGGGAAATATAATTAACGTGCACAGACATAAATTG
KF517305.1      GAGGCATTGGCAGCAAGTGTAAAGATGGGAAATATAATTAACGTGCACAGACATAAATTG
                ************************************************************

NORM            GGTGCACTTAATAAAGCTGTTAAAAAGGTTACGAATGATGAATCGGGTAGTGAAGTTAGC
KF517305.1      GGTGCACTTAATAAAGCTGTTAAAAAGGTTACGAATGATGAATCGGGTAGTGAAGTTAGC
                ************************************************************

NORM            AGCGAGGATGAAGAAAGTGACCAGGAAACCTTACTGCGTAACAGAAAAATGCCAACAAAT
KF517305.1      AGCGAGGATGAAGAAAGTGACCAGGAAACCTTACTGCGTAACAGAAAAATGCCAACAAAT
                ************************************************************

NORM            TCCAAGACGAGAAGCCAGCTCTTTAGAGCGCTCAAAGATTTAAACAAAAGAACCAACCAG
KF517305.1      TCCAAGACGAGAAGCCAGCTCTTTAGAGCGCTCAAAGATTTAAACAAAAGAACCAACCAG
                ************************************************************

NORM            TACTCGGTCAAGCCGGAAAAGGTTTTGGAATATAGCGAGGCCACCAAAGGCAAGAGGATG
KF517305.1      TACTCGGTCAAGCCGGAAAAGGTTTTGGAATATAGCGAGGCCACCAAAGGCAAGAGGATG
                ************************************************************

NORM            TCGGCAGGGTCTAAATTAATCAGCGCCATGACGGTTATACCTTTGCTGACTATATTGTTT
KF517305.1      TCGGCAGGGTCTAAATTAATCAGCGCCATGACGGTTATACCTTTGCTGACTATATTGTTT
                ************************************************************

NORM            TTCATAATTGTTGGTAGCAGCACCATCACAGAAATTAAATCTCACTTAGTACTAAAAGGA
KF517305.1      TTCATAATTGTTGGTAGCAGCACCATCACAGAAATTAAATCTCACTTAGTACTAAAAGGA
                ************************************************************

NORM            CATGACCCAAACGATATACCAACATTGTGTATTGCAACTTACAGTTTGAACTGGTTTGTA
KF517305.1      CATGACCCAAACGATATACCAACATTGTGTATTGCAACTTACAGTTTGAACTGGTTTGTA
                ************************************************************

NORM            TTAATCATGTGTGTGTTGCAAAATTTGATACGGAGTGCACCAATAATAAGCCATGCACTG
KF517305.1      TTAATCATGTGTGTGTTGCAAAATTTGATACGGAGTGCACCAATAATAAGCCATGCACTG
                ************************************************************

NORM            AAGGGTATTGACCGAGACATAAAAGAAGCATATATGAAAAAGGCTGCAGAGGATGACGAG
KF517305.1      AAGGGTATTGACCGAGACATAAAAGAAGCATATATGAAAAAGGCTGCAGAGGATGACGAG
                ************************************************************

NORM            GATGAAGACTAA
KF517305.1      GATGAAGACTAA
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ORF 111

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NORM            TTATACAATTGGCAGCCATCTTCCTTGGCCAACGCCTATAAAATATATTGTGATTGCAGG
KF517297.1      TTATACAATTGGCAGCCATCTTCCTTGGCCAACGCCTATAAAATATATTGTGATTGCAGG
                ************************************************************

NORM            CCACCACAAGAAACACAGCGTCAGCAAATATCTTGCTATAGTAGAAAACAATCCAGGTTT
KF517297.1      CCACCACAATAAACACAGCGTCAGCAAATATCTTGCTATAGTAGAAAACAATCCAGGTTT
                ********* ************************************************

NORM            CTCAATAGGTTCGTGTTTCAACCTGTCTGCCTTGATTAACATCTGTGTTGATTCATCATG
KF517297.1      CTCAATAGGTTCGTGTTTCAACCTGTCTGCCTTGATTAACATCTGTGTTGATTCATCATG
                ************************************************************

NORM            GTCGGGAATGTCTAACGCATCTCTCTTTAACTGTTCGGCTCTCATGATTCTGGTGAACGA
KF517297.1      GTCGGGAATGTCTAACGCATCTCTCTTTAACTGTTCGGCTCTCATGATTCTGGTGAACGA
                ************************************************************

NORM            TGGTAGAATGCTGGTAGAATAACTGATTGCCAATATACCACAAACAAATCCCCAGCTGAT
KF517297.1      TGGTAGAATGCTGGTAGAATAACTGATTGCCAATATACCACAAACAAATCCCCAGCTGAT
                ************************************************************

NORM            GTTCAATGTCAGTTTTGCATGGCAGTCTTTGTAGTAATCAAAATCTGATATGATCGATAT
KF517297.1      GTTCAATGTCAGTTTTGCATGGCAGTCTTTGTAGTAATCAAAATCTGATATGATCGATAT
                ************************************************************

NORM            GACAATGAATGCCACTGACCCTGCTAAGGTTAAAAATAATAAAAACAACGTGATTCCTAT
KF517297.1      GACAATGAATGCCACTGACCCTGCTAAGGTTAAAAATAATAAAAACAACGTGATTCCTAT
                ************************************************************

NORM            AAGAAATCTCACAAAGGTCGTTCCTCTCTTTCCAGATTTCATCCTGCATATATTTGCTAT
KF517297.1      AAGAAATCTCACAAAGGTCGTTCCTCTCTTTCCAGATTTCATCCTGCATATATTTGCTAT
                ************************************************************

NORM            ACAAACGATGATGAATACACCAATCACTACTCCCACAATTGCTATTGTAATTATGGCAGG
KF517297.1      ACAAACGATGATGAATACACCAATCACTACTCCCACAATTGCTATTGTAATTATGGCAGG
                ************************************************************

NORM            GTTGTGTATATACAGTGTTGATTCCATGTGTATCGGTCTTTCACTGTAATCATTACCCTT
KF517297.1      GTTGTGTATATACAGTGTTGATTCCATGTGTATCGGTCTTTCACTGTAATCATTACCCTT
                ************************************************************

NORM            GACCTCAGAGCATAATTTACAGCAGAACTCATCACCTCTGCATTCTTTAACCGGACAAGC
KF517297.1      GACCTCAGAGCATAATTTACAGCAGAACTCATCACCTCTGCATTCTTTAACCGGACAAGC
                ************************************************************

NORM            AGGTGTTTTTACGGGTTTAATTGTGGTTGTGGTGAGTGTACCATTTTCATCCTCTGTTGA
KF517297.1      AGGTGTTTTTACGGGTTTAATTGTGGTTGTGGTGAGTGTACCATTTTCATCCTCTGTTGA
                ************************************************************

NORM            ATTTTCCGTAACTTTATATATGTCTTGCATCTCACATCCACCCATGTGATTACAGGCAGG
KF517297.1      ATTTTCCGTAACTTTATATATGTCTTGCATCTCACATCCACCCATGTGATTACAGGCAGG
                ************************************************************

NORM            TTTATCATAAAGAATGTTGTAGTACTGCAACATAAAGATGCTGAATCCCACAGCACATAT
KF517297.1      TTTATCATAAAGAATGTTGTAGTACTGCAACATAAAGATGCTGAATCCCACAGCACATAT
                ************************************************************

NORM            TAAATGTAGTACCACAATCGGACCAATCAT
KF517297.1      TAAATGTAGTACCACAATCGGACCAATCAT
                *****************************
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APPENDIX D

List of Publications and Conference Proceedings

Publications:

1. **Burioli E.A.V.**, Prearo M., Riina M.V., Bona M.C., Fioravanti M.L., Arcangeli G., Houssin M. (2016). Ostreid herpesvirus type 1 genomic diversity in wild populations of Pacific oyster *Crassostrea gigas* from Italian coasts. *Journal of Invertebrate Pathol*ogy, 137: 71-83. doi: 10.1016/j.jip.2016.05.004.

2. Oden E., **Burioli E.A.V.**, Trancart S., Pitel P.H., Houssin M. (2016). Multilocus Sequence Analysis of *Vibrio splendidus* related-strains isolated from blue mussel *Mytilus* sp. during mortality events. *Aquaculture*, 464: 420-427. doi: 10.1016/j.aquaculture.2016.07.024.

International Conferences:

1. **Burioli E.A.V.**, Scanzio T., Righetti M., Foglini C., Serracca L., Prearo M. (2016). Poster: "Italian seawaters as suitable environment for Pacific oyster *Crassostrea gigas* pre-fattening". *Aquaculture America, New Orleans (USA), February 19th-22nd, 2015.*

2. **Burioli E.A.V.**, Oden E., Trancart S., Le Bas C., Houssin M. (2016). Poster presentation: Multilocus Sequence Analysis: a powerful tool for the classification of *Vibrio splendidus* related strains. Implementation during mussel mortality events in France. *International Vibrio conference, Roscoff (France), March 29th- April 1st, 2016.*

3. **Burioli E.A.V**, Bona M.C., Pastorino P., Fioravanti M.L., Riina M.V., Houssin M., Prearo M. (2016). Poster presentation: Prevalence and phylogenetic analysis of OsHV-1 in wild populations of Pacific oyster *Crassostrea gigas* in Italy. Aquatic Animal Epidemiology, Oslo (Norway), September 20th - 22nd, 2016. *Front. Vet. Sci. Conference Abstract:* doi: 10.3389/conf.FVETS.2016.02.00012.

National Conferences:

1. **Burioli E.A.V.**, Houssin M., Varello K., Gorla A., Fioravanti M.L., Prearo M. (2015). Poster presentation: Isolamento di un membro della famiglia *Flavobacteriaceae* in ostriche concave *Crassostrea gigas* durante un evento di mortalità nella laguna di San Teodoro (OT). *XXI Convegno Nazionale SIPI Auditorium comunale San Nicolò, Chioggia (VE), 8-9 Settembre 2015, Atti p.40* .

2. **Burioli E.A.V.**, Prearo M. , Pastorino P. , Scanzio T. , Gorla A., Houssin M. (2015). Poster presentation: "Descrizione di un evento di mortalità ed indagine diagnostica in ostriche concave adulte allevate nello stagno di San Teodoro (Olbia-Tempio)". *IV Convegno Nazionale S.I.R.A.M., CHIOGGIA Centro Direzionale ASPO – zona porto, Venerdì 6 novembre 2015, Atti p.30-31*.

3. **Burioli E.A.V**., Riina M.V., Colussi S., Pastorino P., Menconi V., Prearo M., Acutis P.L. (2015). Poster presentation: "Identificazione genetica dei banchi naturali di ostrica concava dell'alto-medio Adriatico". *IV Convegno Nazionale S.I.R.A.M., CHIOGGIA Centro Direzionale ASPO – zona porto, Venerdì 6 novembre 2015, Atti p.32-33.*

4. Caldaroni B., **Burioli E.A.V.**, Magara G., Foglini C., Dörr A.J.M., Scoparo M., Elia A.C., Abete M.C., Scanzio T., Prearo M. (2015). Poster presentation: "Biomarcatori di stress ossidativo per il monitoraggio di fenomeni di stress nell'ostrica concava (*Crassostrea gigas*) in differenti siti produttivi marini". *IV Convegno Nazionale S.I.R.A.M., CHIOGGIA Centro Direzionale ASPO – zona porto, Venerdì 6 novembre 2015, Atti p.40-41*.

5. **Burioli E.A.V.**, Houssin M., Bona M.C., Fioravanti M.L., Riina M.V., Prearo M. (2016). Conference presentation: *Ostreid herpesvirus tipo 1* e *Vibrio aestuarianus*, studio di due importanti patogeni dell'ostrica concava nel contesto italiano. *XXII Convegno nazionale della Società Italiana di Patologia Ittica, San Michele all'Adige (Italia), 8-9 settembre 2016*.

6. **Burioli E.A.V.**, Oden E., Trancart S., Prearo M., Houssin M. (2016). Conference presentation: "Sviluppo di una multilocus sequence analysis per l'identificazione dei *Vibrio* appartenenti al clade Splendidus e applicazione della metodica durante un evento di mortalità in molluschi". *XXII Convegno nazionale della Società Italiana di Patologia Ittica, San Michele all'Adige (Italia), 8-9 settembre 2016.*

7. **Burioli E.A.V**., Varello K., Gorla A., Bozzetta E., Prearo M. (2016). Poster presentation: Valutazione di una possibile gametogenesi in individui triploidi di ostrica *Crassostrea gigas*. risultati del primo anno di studio. *XXII Convegno nazionale della Società Italiana di Patologia Ittica, San Michele all'Adige (Italia), 8-9 settembre 2016.*

8. Squadrone S., Pastorino P., Prearo M., Serracca L., **Burioli E.A.V.**, Stella C., Foglini C., Ercolini C., Abete M.C. (2016). Poster presentation: Determinazione del contenuto in metalli nei prodotti d'acquacoltura: un caso studio. *XXII Convegno nazionale della Società Italiana di Patologia Ittica, San Michele all'Adige (Italia), 8-9 settembre 2016.*