Influence of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by *Vibrio harveyi*: an example of emerging vibriosis linked to global warming.

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ABSTRACT

Since 1998, Haliotis tuberculata mass mortalities regularly occur in wild abalone populations in France during their reproductive period and in conjunction with seawater summer temperature maxima and Vibrio harveyi presence. To confirm the importance of bacterial exposure, temperature and reproductive status on abalone susceptibility, experimental infections via bath exposure were performed using abalone ranging from immature to reproductively mature. Ripe abalone were more susceptible to the bacterium than immature specimens ($p<0.001$), and only one degree difference in temperature had a highly significant impact on the mortalities ($p<0.001$). The natural mortalities that were surveyed during summer 2007 confirmed that recent epidemic losses of European abalone appeared in conjunction with host reproductive stress, elevated temperatures and presence of the pathogen Vibrio harveyi. In view of the elevation of the mean summer temperatures observed in Brittany and Normandy over the last twenty-five years, this temperature-dependent vibriosis represents a new case of emerging disease associated with global warming.

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INTRODUCTION

Broadcast spawning invertebrates with high fecundities and larval dispersal were thought to be invulnerable to extinction, but evidence for decline and local disappearance of marine species is becoming more and more evident (Jamieson, 1993; Myers & Worm, 2003). Abalone are an example of this global decline, and white and black abalones (*Haliotis sorenseni* and *H. cracherodii*, respectively) are listed as endangered species in the USA (Gruenthal & Burton, 2008; Hobday *et al.*, 2001). Many authors suggest that different factors contribute to fish and shellfish decline including

over-exploitation of the resource, inadequate enforcement policies, diseases and environmental changes (e.g. Vilchis *et al*., 2005, Rothaus *et al*. 2004, Altstatt *et al.*, 1996)
Temperature elevation, due to global change, can directly influence marine populations by inducing physiological stress (Li *et al.*, 2007; Przeslawski *et al.*, 2005) or by disturbing natural reproduction cycles (Lawrence & Soame, 2004). In addition to impacting macrofauna, global warming also influences the microbiota, for instance through changes in the infectivity of potential pathogens (Rosenberg & Falkovitz, 2004). Recent reviews have emphasized the importance of parasites and pathogens on terrestrial and marine taxa in the framework of the global warming (Bally & Garrabou, 2007; Ward *et al.*, 2006; Harvell *et al.*, 2002).
European abalone, *H. tuberculata,* stocks are in decline (Huchette & Clavier, 2004), and numerous recent mortality episodes in both farmed and wild populations have been associated with the protozoan parasite *Haplosporidium montforti* (Azevedo *et al.*, 2006a; Balseiro *et al.*, 2006), rickettsia-like bacteria (Azevedo *et al.*, 2006b; Balseiro *et al.*, 2006) or microbes of the genus *Vibrio* (Nicolas *et al.*, 2002). During the mass mortality episodes of September 1998 and 2000 in France, *Vibrio carchariae* strains (junior synonym of *V. harveyi* (Gauger & Gomez-Chiarri, 2002)) were isolated from farmed populations and wild *H. tuberculata* (Nicolas *et al.*, 2002). This Gram-negative bacterium was already known to cause shellfish mortalities (Austin & Zhang, 2006; Aguirre-Guzman *et al.*, 2004; Nicolas *et al.*, 2002; Goarant *et al.*, 2000; Nishimori *et al.*, 1998; Liu *et al.*, 1996) and gastroenteritis in fish (Lee *et al.*, 2002).
The French abalone mortalities are always recorded, both in the natural and farmed stocks, at the end of summer, when seawater temperatures are maximal. A correlation between local maximum temperature and the occurrence of these mortalities was proposed by Huchette and Clavier (2004). Moreover, the abalone reproductive cycle appeared as an important factor associated with mortalities (Nicolas *et al.*, 2002; Nishimori *et al.*, 1998). Numerous diseases are known to be linked to reproduction. The best example is the oyster *Crassostrea gigas* "summer mortalities" that have been linked to energy deficiency during the reproduction period (Delaporte *et al.*, 2007; Samain *et al.*, 2007; Perdue *et al.*, 1981).
In the present work, (1) laboratory experiments to test the influence of various conditions (temperature, gonad condition and age/size) on disease development as well as (2) field surveys during mortality periods, where different parameters were recorded (mortality percentage, temperature and reproductive status) were performed. This to examine the factors influencing the interaction between *V. harveyi* and *H. tuberculata* in an attempt to better understand the emergence of disease in this model.

MATERIAL AND METHODS

A. Laboratory experiments

1. Biological material

Abalone
For all laboratory experiments, animals were kept in aerated 5 L (young adults) or 40L (adult) static seawater tanks. Acclimation of young animals was systematically performed in our laboratory animal facilities by introducing the specimens in seawater at "France Haliotis" hatchery/environmental temperature. To avoid shock, temperature was raised with 1°C per 2 days until experimental temperature was reached; subsequently animals remained at least 3 days at this temperature before experimentation start. During the conditioning (see below) of adult animals in the SMEL facility abalone were fed *ad libitum on a* marine algae diet of *Gracilaria* sp. and *Palmaria* sp.
Seawater was renewed every day and temperature was recorded using an electronic thermometer (±0.1 °C).
Please note that of all abalone introduced into the LEMAR or SMEL facilities, only one batch called "Post-spawning Batch" from the adult abalone conditioning experiment (see below) presented about 20% mortalities between the collection date and the beginning of the experiments.
Young adults. Immature (n=200, 29.0 mm ± 1.6, 1 ½ years old) abalone were transferred in March 2006 from the "France Haliotis" hatchery (Plouguerneau, France) to the LEMAR laboratory; ripe

abalone (n=500, 35.0 mm ± 2.1, 2 years old) were introduced in August 2006. Note that both groups came from the similar broodstock fertilization but were taken at different periods of the year.

Adults. Adult abalone (n=240, 70.0 mm ± 15.4 mm, >4 years old) were collected by scuba diving in the North Cotentin (near Fermanville, Normandy, France) in June 2006 and transferred to the experimental marine station of the SMEL. Immediately after collecting, 2 groups of 200 abalone were put in two 500L tanks for a 3 days antibiotics treatment (Flumiquil 10% - 80 g/m^3 and Adjusol TMP Sulfa liquid 50 ml/m^3) to prevent bacteria-associated mortalities. After this disinfection phase, gonad ripening was induced.

2. Inducing gonad ripeness in adult abalone

To obtain abalone at different maturation stages for use in bacterial infection trials, the two tanks of antibiotic treated adult abalone (described above) were maintained for one month under two different temperature conditions (Figure 1) (Hahn, 1994). Tanks were aerated and 20% of the seawater was daily renewed. One batch, called "Ripe Batch", was kept in 13°C seawater to obtain a gonad maturation at stage 4 and 5 (ready to spawn) at the time of bacterial infection. The other batch, called "Post-spawning Batch", was maintained at 18°C to obtain spending abalone (stage 6) at the time of bacterial infection (Figure 1). Note that, in this group about 20% of the animals died. No *V. harveyi* could be isolated (as the animals were antibiotic-treated in the SMEL hatchery). This mortality is often observed when large wild and ripe animals are introduced into the hatchery; probably caused by the fishing, antibiotics treatment and the slow (1°C/2 days) augmentation of the temperature.

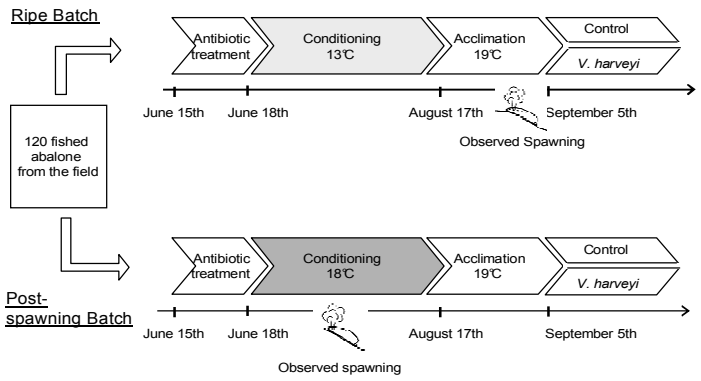

Figure 1: Experiment design. The 120 fished abalone were treated by antibiotic, conditioned for 2 months at 13°C ("Batch ripe") or 18°C ("Batch post-spawning"), acclimated at 19°C before bacterial challenge, as described in material and methods. Observed spawning are indicated by a picture.

To confirm the maturation status of abalone, histological sections were performed on at least ten animals per condition and gonad development stages were determined as described below (see section B.4. *Gonadal development determination by histology*). At the end of the conditioning, groups of 20 abalone were transferred into aerated 40L tanks before bacterial infections.

3. Bacterial experimental test – General protocol

In order to determine the influence of several stressors on the susceptibility of abalone (in varying stages of maturation) to *V. harveyi* infection a standard 24 hr bath exposure protocol in conjunction with a single stressor was used. This standard protocol was adapted from Nicolas *et al.* (2002). The virulent *Vibrio harveyi* strain, ORM4, isolated in 2002 from infected *H. tuberculata* in Normandy (France) (Nicolas *et al.* 2002), was used for all experimental tests. Bacterial cells were grown for 18h at 28°C in Luria-Bertani Broth complemented with extra salt (10g/l, LBS), centrifuged, washed twice in filtered sterilized seawater (FSSW), and resuspended in FSSW. This bacterial suspension was inoculated at 10^5 cell ml^{-1} (final concentration) into seawater tanks with 10 young abalone. All abalone which died in these trials were ground in FSSW with an ultra-turrax homogenizer. The tissue suspension was gently centrifuged (600g , 10min at RT) and filtered (subsequently over a 100, 50 and 20 µm mesh to remove tissue debris. The filtrate was used as bacterial inoculum, and added at about 10^5 CFU/ml, (which was checked by plating), into 5 L tanks (for young abalone) or 40 L tanks (for adults) containing filtered (0.5 µm) seawater. For each experiment, triplicate groups containing each

20 abalone per tank, were used. Seawater was renewed and temperature was monitored each day. Dead abalone were counted and removed twice daily.
A concise version of all these experimental details, batches and conditions is presented in Table 1. Please note, that the infections of young and adult abalone was done at the same date in August with exactly the same bacterial inoculum (sent by special delivery), except for the young immature abalone that was tested in March.

Age	Batch	N*	Size	Maturation Stage	Lab Conditioned	Source	Experiment
Young	" immature"	200	29.0 ± 1.6	Immature	No	Hatchery	Ripeness effect
	"ripe"	500	35.0 ± 2.1	Ripe	No	Hatchery	Temperature effect Ripeness effect Size-age effect
Adults	"post-spawning"	120	70.0 ± 15.4	Stage 3	Yes	Field	Ripeness effect
	"ripe"	120	70.0 ± 15.4	Stage 4-5	Yes	Field	Temperature effect Ripeness effect Size-age effect

* Number of abalone per batch

Table 1: *Haliotis tuberculata* experimental batches and conditions.

a) Temperature effect

To understand the relationship between seawater temperature and abalone susceptibility to *V. harveyi* infection, the animals were exposed to the bacterium (with the standard protocol described in section A.3) under a range of temperatures that reflect the range of summer temperatures recorded for north-western France (see section B.1. below). In the first experiment, 2 yr old, farm-raised, mature abalone were infected with bacteria at 15°C, 17°C, 18°C, 19°C or 20°C in the 24 hr bath exposure method described above and followed over 10 days. In a second experiment, wild-caught, adult abalone from the "Ripe Batch" (see section A.2. *Inducing gonad ripeness in adult abalone*) were infected at the 15°C, 17°C or 19°C and monitored for 22 days.

b) Ripeness effect and age-size effect

To determine the influence of the reproductive maturation stage and of abalone size, the following four groups of abalone were infected with bacteria for 24 hr and held at 19°C: – 1. Large "Ripe Batch" adults (70 mm, stage 4-5); 2. Large "Post-spawning batch" adult (70 mm, stage 6); 3. Farmed young ripe adults (35 mm) and 4. Farmed young immature adults (29 mm). Mortalities were monitored on daily basis throughout the 10 days (young abalone) or 22 days (adult) trials.For husbandry practices, see section A3. *Bacterial experimental test-general protocol.*

B. Field survey

1. Temperature

From 1991 to 2006, mean daily values of seawater temperature in Flamanville (Cotentin, Normandy, France) were obtained from the French Energy Company, EDF (CNPE Flamanville). Seasonal means were calculated.
Temperature was registered every hour over the survey period at the Bretteville site (Cotentin, Normandy, France). Probes were placed next to the pipe containing our group of abalone (see section B.2.). Daily means were calculated.

2. Animal and seawater sampling

Eighty abalone were collected by scuba diving at the Bretteville site (Cotentin, Normandy, France) from July 2007 to November 2007 (10 abalone at each sampling date). The site was chosen in the subtidal area at 6 meters below sealevel to avoid "too easy abalone fishing". The numbers of dives per month varied, and were adapted to mortality predictions: July (n=1 dive), August (n=2), September (n=3), October (n=1), November (n=1). Seawater (100 mL) was also collected at 1m of depth and at the seawater-sediment interface using 100 mL syringe by the divers. These samples were transported on ice to the laboratory and bacterial concentrations were determined (see section B.5. *Bacterial count and identification*).
In July 2007, 50 abalone were also collected from this same site and transferred into an "abalone culture container" that consisted of a polyethylene pipe measuring 70 cm in length and 47 cm in diameter of which one end was closed with an inox cover. After introducing the abalone and local kelps, the open end of the pipe was covered with a 6 mm mesh Nytex screen, and the pipe was attached to a concrete ballast. The animals were verified at approximately weekly intervals from July to November, and dead and live animals were counted. Haemolymph from moribund abalone or from those which appeared to have recently died was aspirated with a 25 G needle and 2 ml syringe. Aliquots (100 µl) and muscle homogenates were diluted and plated on thiosulfate-citrate-bile salts-sucrose agar (TCBS, a vibrio selective medium, AES Laboratory) and incubated at 28°C. The remainder of these tissues was frozen at -20°C for later testing of the pathogen.

3. Biometry and condition index

In the laboratory, each abalone was removed from its shell and the pedal-adductor muscle was separated from the viscera. Abalone length, wet and dry (80°C for 48 h) weights of the viscera were measured. Visceral (VCI) condition indices were calculated as follows:
VCI = Organ dry weight*100/shell length^3

4. Gonadal development determination by histology

Histology examination was used to characterize the reproductive maturation of individual abalone. A 5 mm section of the viscera that contained gonad and digestive tissues was weighed, fixed in Bouin's solution (Bancroft and Stevens, 1982) for 48h, dehydrated in an ascending series of ethanol and embedded in paraffin. Sections of 5 µm thickness were stained with Masson's trichrome (Bancroft and Stevens, 1982). Each histological section was examined using light microscopy. Animals were assigned into one of the six gonadal developmental stages according to Sobhon (1999). Stage 1 corresponds to the pre-proliferative stage (gonad is essentially undifferentiated with little or no germinal epithelium between the outer mantle epithelium and the digestive gland), stage 2 corresponds to the proliferative stage (oogonia form clusters on trabecular walls and become stalked at about 10 µm), stage 3 corresponds to the advanced development stage (an early ripening ovary is rized by newly stalked oocytes measuring about 25 µm, vitellogenesis is initiated during this stage), stage 4 corresponds to the ripe stage (ovary has oocytes measuring >110 µm, mature oocytes are free from the trabeculae), stage 5 corresponds to a partially spawned animal (ovary contains a reduced density of mature oocytes relative to ripe gonads), stage 6 is the spent stage (characterized by lack of ripe gametes and slight gametogenic activity).
For each female, 10 randomly chosen fields were selected from which the area of about 150 individual mature oocytes (with clearly visible nucleoli to ensure that each section passed through the centre of the gamete) was measured using Image-Pro Express v6.0. Theoretical diameters were then calculated:
Theoretical diameter = Square root (4*Surface/pi).

5. Bacterial count and identification

To detect the presence of *V. harveyi* in abalone (within haemolymph or muscle tissues) or in seawater (from the water column or at the seawater-sediment interface), aliquots were serially (10 fold) diluted

in FSSW and 0.1 ml was spread onto three types of media. These included Luria-Bertani Broth (LB, Sigma with 10g.L^{-1} NaCl, LBS), Vha (*V. harveyi* specific medium; Harris *et al.*, 1996) and TCBS. Plates were incubated at either 18°C (LBS) or 28°C (Vha and TCBS) to reflect ambient seawater temperatures and culture optima for this bacterium, respectively.
Colonies with *V. harveyi* phenotype on Vha plates and on TCBS (light green dense) were isolated. and purified three times. Bacteria were cultured overnight in LBS broth at 28°C and DNA was extracted using a traditional phenol-chloroform method (Sambrook *et al.*, 1989). DNAs isolated from *V. halioticoli* (strain LMG19700) and *V. harveyi (strain* ORM4) were used as negative and positive control for PCR, respectively. The 16S rDNA was amplified with the following universal eubacterial primers and reaction conditions (Kalmbach *et al.*, 1997): Forward primer – 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer – 5' CGGYTACCTTGTTACGAC 3'. ToxR DNA was amplified with specific primers Vh-toxR-F 5'-TTCTGAAGCAGCACTCAC 3' and Vh-toxR R 5'-TCGACTGGTGAAGACTCA 3' and the reaction conditions of Conejero & Hedreyda (2003). PCR products were separated on a 1.0% agarose gel with 0.1 µg/mL ethidium bromide and visualized on a UV transilluminator. 16S rDNA PCR products were sequenced and compared to the public sequences in the NCBI databases using the BLAST algorithm.

C. Statistical analyses

Comparison of survival curves were performed with a Kaplan-Meier analysis. The details of this comparison are given in table 2.
Correlation between abalone size (length) and day of death was estimated with the Pearson correlation coefficient. Differences were considered to be statistically significant at $p < 0.05$ and correlation was retained when $p < 0.1$.
Statistical analyses were performed using the MedCalc® (version 9.6.4.0) statistical software.

RESULTS

A. Evidence of seawater temperature increase in Normandy

From 1991 to 2007, the mean summer seawater temperatures in Normandy increased from 16.1 to 17.5°C. A linear regression through these data provides us with a 1.5°C increase over a 17 years period (Figure 2).

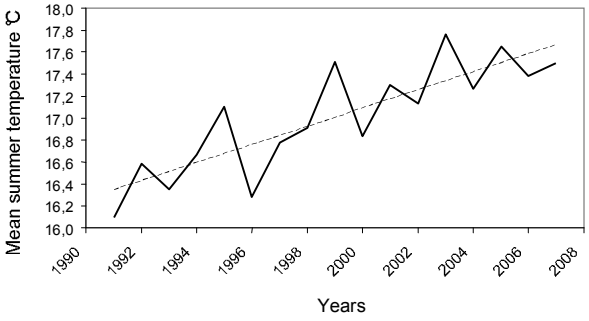

Figure 2: Flamanville (Normandy) summer mean water temperature (in °C). Trend is indicated by the dot line.

B. Factors impacting abalone susceptibility to V. harveyi: an experimental approach

Temperature effect
Experimental infections of young "naturally riped" abalone were done at five temperatures (15°C, 17°C, 18°C, 19°C and 20°C). Within 5 days no mortalities were detected in the 15 and 17°C containers. However, when temperatures were 18°C or higher, 80% of the abalone died (Figure 3 and Table 1, Kaplan-Meier p <0.0001). Similar results were obtained with ripe adult abalone: 0% mortality at 15°C and 17°C, and 83% mortality at 19°C after 20 days (data not shown).

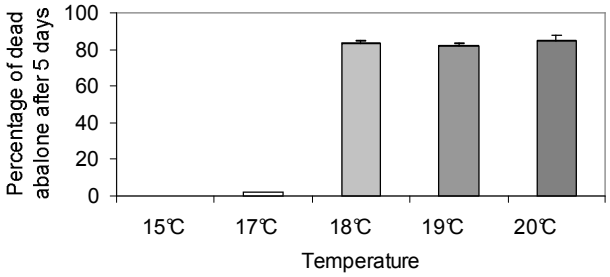

Figure 3: Temperature influence on *V. harveyi* associated mortalities. Young ripe abalone were exposed at 15°C, 17°C, 18°C, 19°C or 20°C, by immersion to 10^5 bacteria/ml during 24h. Seawater was changed daily. Dead abalone were counted twice a day. Bars show standard error.

Age and size effect

Mortalities after bacterial infections at 19°C were estimated in adult and young ripe abalone. Cumulative losses between young ripe (80%) and adult abalone (75%) were similar (Figure 4). However, young ripe abalone died between days 5 and 10, while for the older ripe animals death occurred over a longer period. This results in statistically significant differences between the survival curves when tested by Kaplan-Meier analysis ($p<0.0001$, Table 2.). Within the group of mature abalone no significant correlation between size and day of death could be detected (Pearson, p =0.189, n=72).

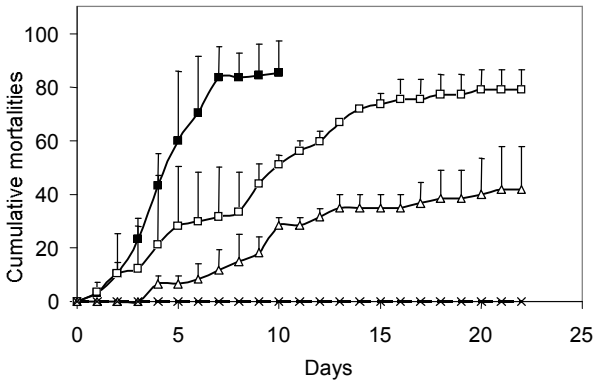

Figure 4: Age, size and gonad maturation influence on *V. harveyi* associated mortalities. Abalone were exposed by immersion at 19°C to 10^5 bacteria/ml during 24h. Seawater was changed daily. Dead abalone were counted and removed twice a day. Bars show standard error. Black boxes correspond to 2-years old ripe abalone from hatchery. White boxes to >4-years old ripe abalone from field. White triangle to >4-years old non-ripe abalone from field. Crosses to controls without bacteria.

Ripeness effect

After bath exposure with *V. harveyi*, up to 85% of the ″Ripe Batch″ abalone (partially spawned at the beginning of the experiment, stage 4-5) died within 15 days, whereas only 40% of the ″Post-spawning Batch″ abalone (stage 6) died during this period (p = 0.001, Figure 4 and table 2.). In addition, immersion tests using young abalone in which gonads were in an advanced but not yet fully mature stage (March 2006, stage 3) failed to induce mortality in presence of *V. harveyi* (0% mortality after 3 weeks and 2 consecutives infections - data not shown).

C. Field survey

Natural mortalities

Losses of abalone placed in the culture pipes in Normandy (France) occurred during summer 2007 between August 28th to October 2nd. Cumulative mortality from July till November reached 63% (Figure 5C).

Spawning

Microscopy examination of stained tissue sections revealed evidence of reproductive development (between July 18th and September 4th) and release of mature oocytes (between September 4th and 11th, Figure 5A). Oocytes reached the maximum size of 124 µm (Figure 5: Image A5 on September 4th) before being released from the gonad (Figure 5: Image A6 on September 11th). Between September 4th and 11th, a decrease in visceral condition index (VCI) was observed in conjunction with histological evidence of spawning (Figure 5A, B). This period coincided with the observed abalone mortalities.

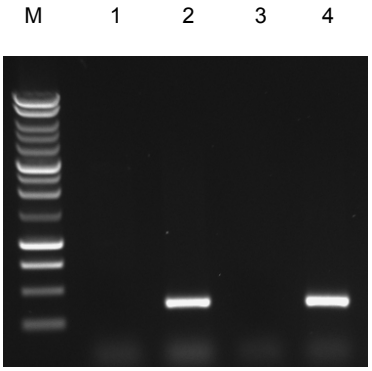

Figure 6: Amplified fragments produced in toxR PCR Line 1, 2 and 3 controls from with distilled water, *V. harveyi* ORM4 DNA and *V. halioticoli* DNA. Line 4 corresponds to strain 07023 isolated from haemolymph of moribund abalone in 2007 in Normandy (France). Lane M is a 1kb marker (Promega).

Seawater temperature
Seawater temperature in Normandy varied from 14.4°C to 17.9°C during the survey period (mean 16.8°C ± 0.9°C). Spawning occurred when seawater temperature was maximal (17.9°C) (Figure 5D), and mortality started to occur.

Microbial patterns
Total presumptive vibrio counts, contained in the water column and at the seawater-sediment interface, changed over the course of the study. At the seawater-sediment interface, presumptive vibrios concentrations were high (mean of 6×10^6 CFU/ml during the survey), and reached their maximum in September (4×10^7 CFU/ml, Figure 5D). Similar trends in presumptive Vibrio counts were quantified from the water column with a maximum in September (2×10^5 CFU/ml) and a mean during the survey of 4×10^4 CFU/ml. A correlation between presumptive Vibrio concentration near sediments and instantaneous mortalities was found (correlation coefficient = 0.694), even if single regression was not significant ($p = 0.08$) and number of data points was low (n=7).
V. harveyi was isolated from the seawater-sediment compartment in August, September and October. Its colonies were selected using their typical light dense morphology and their green coloration on TCBS plates and their growth on Vha medium. A toxR-specific PCR confirmed this identification. BLAST alignments of the 1500 bp of 16s rDNA sequence confirmed this phenotypical identification.
During the mortality period (see section B *Natural mortalities*) high concentrations of pure cultures of vibrio were isolated from moribund abalone's muscle tissue and haemolymph (mean of 1.1×10^8 CFU/ml haemolymph (s.e. 0.7×10^8 CFU/ml)). These pure cultures from both haemolymph and muscle, corresponded to *V. harveyi*, as evidenced by morphology, growth on Vha, positive amplification with toxR specific PCR (Figure 6, Line 4, strain 07021 from a moribund abalone), and 16s rDNA sequencing.

DISCUSSION

This paper highlights the current and potential future impacts of global warming with respect to increased seawater temperatures, on the host-parasite relationship between *H. tuberculata* and its recently emerged pathogen *V. harveyi*. As abalone populations continue to decline world-wide, additional stressors such as increased temperatures and their influence on host physiology and susceptibility to pathogens will further hinder restoration and management of this economically important shellfish. To illustrate this, Harvell *et al.* (2002) showed that as small as a 1°C temperature elevation could alter a host-parasite relation in favour of the pathogen and lead to epidemic disease. Given these observations, it is important to have a thorough understanding of factors that contribute to abalone disease transmission and expression using a combination of field and laboratory studies as we have conducted with *H. tuberculata* and its vibrio pathogen. Furthermore, implication of gonadal condition and abalone age were also analysed and considered to be involved in the abalone mortality phenomena in Normandy.

The present study tried to associate data of *V. harveyi* associated-mortalities with temperature and gonadal development. We found that mortalities appeared when temperature exceeded 17°C, during the spawning period and only in presence of the pathogenic bacteria *V. harveyi*.

Temperature
Temperature modulates the physiology of poikilothermic organisms including marine invertebrates as well as their pathogens. Many vibriosis are known to be influenced by temperature, including those infecting corals (Banin *et al.*, 2003; Ben-Haim *et al.*, 2003), bivalves (Paillard, 2004a), starfish (Staehli *et al.*, 2008), fishes such as salmon (Colquhoun & Sorum, 2001), and others. Growth, adherence and virulence factor expression of vibrios are directly influenced by temperature (Lin *et al.*, 2004; Johnston & Brown, 2002; Colquhoun & Sorum, 2001; Toren *et al.*, 1998). In addition, host susceptibility to a pathogen may also be influenced as observed in the present study for European abalone face to *V. harveyi.* We demonstrated that the 17°C temperature threshold decides whether

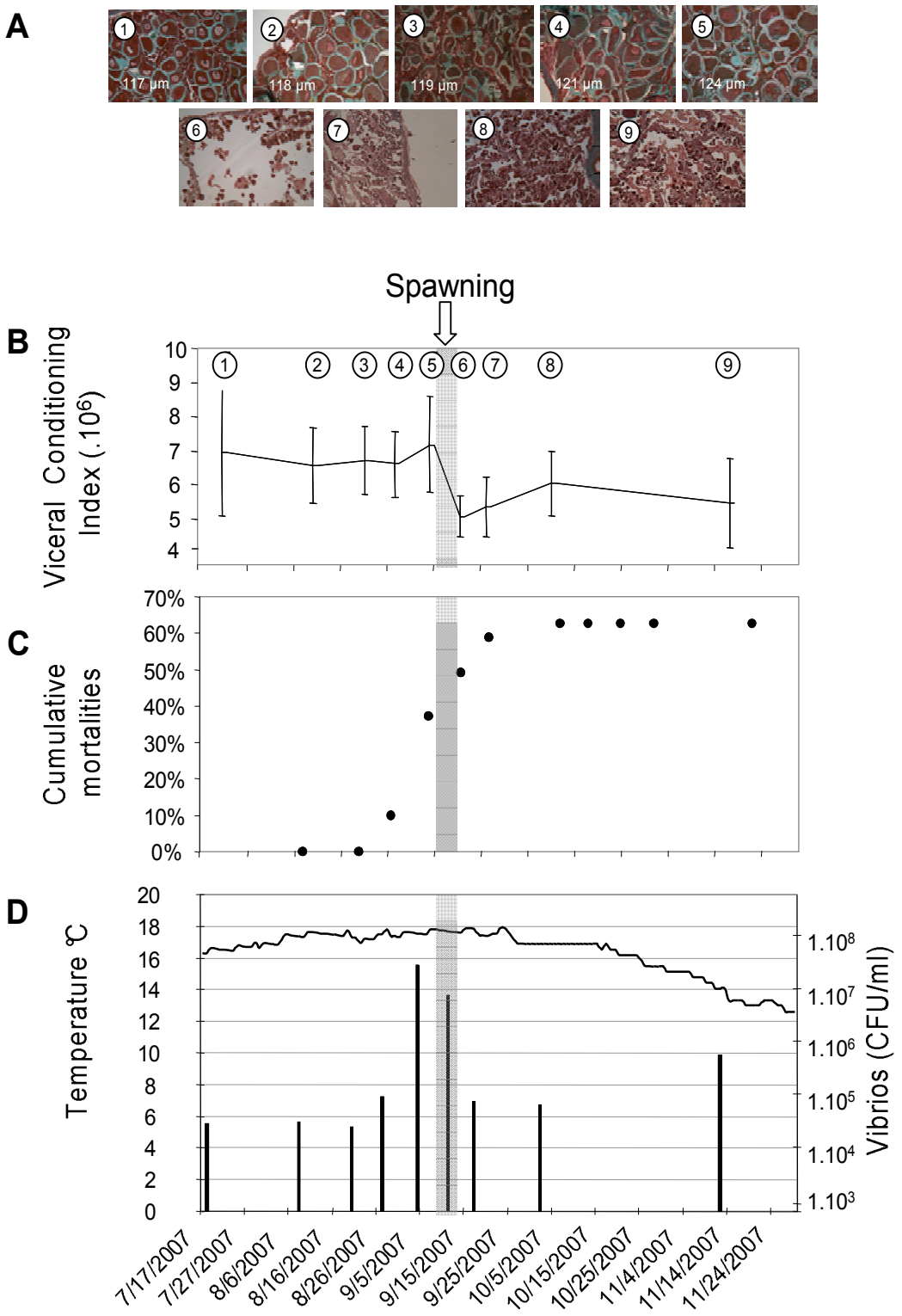

Figure 5: Field survey (July to November 2007)

A. Evolution of ovocytary diameter within the season. Mean diameter of mature oocytes is indicated in the white boxes at the left down corner of picture. Numbers correspond to date ① 7/18, ② 8/07, ③ 8/20, ④ 8/27, ⑤ 9/04, ⑥ 9/11, ⑦ 9/17, ⑧ 10/02 ⑨ 11/12.

B. Evolution of visceral conditional index and estimation of spawning date.

C. Cumulative mortalities recorded on the 50 abalone in a pipe. Grey section corresponds to the estimated spawning date.

D. Seawater temperature and vibrios count in sediment-water interface. vibrions count corresponds to 28°C TCBS grown bacteria. Grey section corresponds to the estimated spawning date.

mortalities occur or not in ripe European abalone: at or below this threshold little or no losses were found. This is in complete agreement with former theoretical (Huchette & Clavier,2004) and laboratory studies (Nicolas *et al.,* 2002). This temperature threshold of 17°C found in our experimental tests has to be placed in relation with measured temperatures for the "Iroise Sea" (Brittany, France) (Esnault & Barraer, 2005) and the Normandy Channel coasts (Fig. 2) that have experienced a summer warming trend of about 1 to 1.5°C over the last 17 years. With this summer temperature augmentation an environmental change occurs from a non-permisive temperature for *V. harveyi infection*, to a temperature where disease can develop. This suggests that seawater warming is a key factor acting in promoting *V. harveyi*-associated mortalities in Normandy and Brittany, France. Similarly, vibriosis of *H. diversicolor supertexta* was also shown to be temperature-dependant (Lee *et al.*, 2001). In Taiwan, this abalone species was also more resistant to *V. parahaemolyticus* infection when temperature was at 24°C than at 28°C or 34°C (Cheng *et al.*, 2004). In addition, Vilchis *et al*. (2005) also showed a negative effect of ocean warming on red abalone (*H. rufescens*) by promoting another fatal bacterial disease, the "withering syndrome".

It is important to consider not only temperature peaks but also the length of exposure to high temperatures. Long periods of high temperatures occurred also in the earliest eighties in France (Southward, 1960), but no large mortalities were reported. Also the apparent absence of mortalities in the Mediterranean and at the Azores is posing a problem.
All this is either due to little interest of the scientific community for this gastropod, or real absence of disease in these areas, either due to the absence of the pathogen, adaptation of the host, or, in northern France, a too short periodicity of warming. Thus far, we know that a 24 hours minimal period of high temperature causes disease development. A report on *H. discus* showed that in Japan in laboratory experiments, 1 hour at 23°C in the presence of 10^5 bacteria/ml (Note, that this is another *V. harveyi* strain) could not kill the abalone. However, 5 hours with these high concentrations were fatal. To understand the importance of long periods of high temperature, such a minimum period for infectivity has to be defined for the *H. tuberculata – V. harveyi* interaction at 18°C.

Establishing a relationship between environmental vibrio concentrations and abalone mortalities furthers our understanding of the host-parasite relationship. Moreover, this enhanced our predictive capabilities enabling forecasting of vibrio-related mortalities. A positive correlation seems to exist between the vibrio "seawater column" or "seawater-sediment interface" concentrations and abalone mortalities recorded in France which appear to occur in the warm-water months. Similarly, a relation between field temperature and vibrio concentration were previously suggested (Gonzalez-Acosta *et al*., 2006; Stabili *et al.*, 2005) as seasonality of infection corresponded to warm-water months with high vibrio concentrations. The concentrations observed in the present study (mean of 6×10^6 CFU/ml (seawater-sediment interface) and 4×10^4 CFU/ml (water column)) are higher than those generally reported in association with animal mortalities (Comeau & Suttle, 2007; Thompson *et al.*, 2004; Pujalte *et al.*, 1999); but reports show extremely variable numbers (Raghunath *et al.*, 2007; Koren & Rosenberg, 2006). The concentration of vibrios is often 80x higher at the seawater-sediment interface than in the water column (Comeau & Suttle, 2007). We observed a similar trend in this study. Given the benthic nature of abalone and their herbivorous grazing behaviour, we would like to suggest that they are more likely to be exposed to lethal doses of vibrio if more bacteria are present in this part of the environment.
As at 17°C the V. harveyi ORM4 could be at its absolute temperature limit, and therefore be unable to infect, we established growth curves from 16 till 19°C (data not shown) and only very little growth rate differences exist. The bacterium growth well at 16°C in complete rich growth media, and therefore we have to hypothesize that low temperature causes an absence of certain virulence mechanisms in the bacterium.
In the environment, low temperatures are known to induce changes in *V. harveyi* physiology leading to the "Viable But Non Cultivable state" (VBNC). The increase to 18°C may resuscitate in the seawater *V. harveyi* from the VBNC non-virulent state to the growing virulent state as described by (Sun *et al.*, 2008). However, to find evidence for this, exact numbers and localizations of *V. harveyi* and vibrios in general in the environment, and not only presumptive vibrios as counted by TCBS plating, are

needed. A Q-PCR analysis, perhaps combined with an extensive bacterial plating experiment, should be established to understand the environmental bacterial cycle in relation with abalone disease.

Temperature can act on bacterial growth and virulence, and also on animal susceptibility to disease by changing its physiological and immunological status (Gagnaire *et al.*, 2006; Paillard *et al.*, 2004b). Stressors are known to modulate immune response (Hooper *et al.*, 2007; Monari *et al.*, 2007; Liu *et al.*, 2004; Malham *et al.*, 2003). Therefore, stress caused by temperature increase and spawning effort, may contribute to render intertidal molluscs more susceptible to disease (Chu, 2000).

Maturation and Spawning

Abalone maturation was the second most important factor directly influencing mortalities caused by *V. harveyi*. In this study, a clear difference in susceptibility to *this bacterium* was observed between ripe and immature animals at 19°C. Immature abalone were insensitive to *V. harveyi*, while ripe (stage 4-5, ripe - partially spawned) or post-spawning (stage 6 spent) abalone were susceptible to infection and mortality. Production of gametes and its elimination are known to be associated with an energy deficit (Dridri *et al.*, 2007; Barkai & Griffiths, 1988). Hayashi (1983) observed a decrease in the glycogen content of muscle during gonad maturation of *H. tuberculata*. Spawning effort has also been well documented as a key factor in summer mortality of *C. gigas* (Delaporte *et al.*, 2007; Royer *et al.*, 2007; Samain *et al.*, 2007; Pouvreau *et al.*, 2003). Recently, Li *et al.* (2007) described the influence of spawning on the thermal resistance of Pacific oysters; post-spawning oyster were shown to presented higher mortalities in response to a temperature shock than pre-spawning oysters. This difference was attributed, in part, to a decrease in production of heat shock proteins and to a reduced immune response (Li *et al.*, 2007). A note of caution should be emitted concerning our laboratory experiments as we stimulated gonad development through a raise of temperature, which directly links temperature with maturation. Experiments where, for instance, food limitation is used to restrict gonad development should help corroborate our findings.

As in other molluscs, abalone gonadal maturation is directly controlled by temperature (Lopez & Tyler, 2000; Girard, 1972) and spawning can be induced by thermal shock. Thus, in the natural environment, spawning and temperature are interrelated. In the present field study, disease appearance was associated with different events: (1) Seawater temperature, as mortalities appeared when temperature was higher than 17°C, (2) Abalone gonad maturation, as mortalities appeared centred on spawning period (2 weeks before and after spawning) and (3) The presence of the pathogenic bacterium *V. harveyi*, that was isolated during these mortalities from the haemolymph of moribund abalone and surrounding seawater.

Age / Origin

The influence of age, size and origin on disease susceptibility is often important in managing disease. In the current study, reproductively mature abalone from both the field and hatchery were susceptible to vibriosis and animal size at least within the field collected animals did not appear to influence susceptibility. We used both wild and farmed abalone to compare susceptibility between both populations under the similar experimental conditions. It is important to note that although we were obliged to use these two locations for their specific facilities, which obligatorily introduced bias, we tried to restrict this to the maximum by using exactly the same bacterial inoculum and infecting them at the same day with the same protocol. Despite large differences in animal size, ~80% of both the farmed and the hatchery abalone died. However, the mortality rate differed between these groups by being faster for the farmed (80% losses in 5 d) than for the wild abalone (~80% losses after 2-3 wks). These kinetics differences can either be attributed to a real age difference, or more likely to size or surface differences, or even their origin (as we are obliged to compare hatchery-grown with wild catch). (Disease expression among farmed mollusc populations is often higher than in wild con-specifics : it can be explained by increased stress or reduced genetic diversity in farmed populations, such as observed in Pacific oysters with “summer mortalities” (Gagnaire *et al.*, 2007)). To understand our « kinetics differences », additional experiments are needed, using for instance, farmed animals of a same age and family group but of different size, or, animals of clearly different ages but of similar size.

In conclusion, this is the first report of a negative effect of global warming on European abalone stocks which influences gonad maturation and the time of spawning (Lawrence & Soame, 2004), probably by influencing abalone immune function (Travers et al., In press) and bloom dynamics of a pathogenic vibrio. The one-degree increase that occurred in France during the last ten to fifteen years seems to be directly responsible for recent *H. tuberculata* mortality outbreaks in Normandy and the North of Brittany. Apparently global warming, through this small, one-degree increase in temperature, largely influences both bacterial and host physiology which understanding demands further study.

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