Risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011

Tracy A. Clegg[a], Teresa Morrissey[b], Fiona Geoghegan[b], S. Wayne Martin[c], Kieran Lyons[b], Seán Ashe[d], Simon J. More[a]

a. UCD Centre for Veterinary Epidemiology and Risk Analysis, University College Dublin, Belfield, Dublin 4, Ireland

b. Marine Institute, Rinville, Oranmore, Co. Galway, Ireland

c. Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

d. Department of Agriculture, Food and the Marine, Kildare Street, Dublin 2, Ireland

Corresponding author: Tracy Clegg, ph +353 1 716 6142, fax +353 1 716 6147

email: tracy.clegg@ucd.ie

Abstract

The Pacific oyster, *Crassostrea gigas*, plays a significant role in the aquaculture industry in Ireland. Episodes of increased mortality in *C. gigas* have been described in many countries, and in Ireland since 2008. The cause of mortality events in *C. gigas* spat and larvae is suspected to be multifactorial, with Ostreid herpesvirus 1 (OsHV-1, in particular OsHV-1 µvar) considered a necessary, but not sufficient, cause. The objectives of the current study were to describe mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated environmental, husbandry and oyster endogenous factors.

A prospective cohort study was conducted during 2010 to 2012, involving 80 study batches, located at 24 sites within 17 bays. All 17 bays had previously tested positive for OsHV1-µvar. All study farmers were initially surveyed to gather relevant data on each study batch, which was then tracked from placement in the bay to first grading. The outcome of interest was cumulative batch-level mortality (%). Environmental data at high and low mortality sites were compared, and a risk factor analysis, using a multiple linear regression mixed effects model, was conducted. Cumulative batch mortality ranged from 2% to 100% (median = 16%, interquartile range: 10% - 34%). The final multivariable risk factor model indicated that batches imported from French hatcheries had significantly lower mortalities than non-French hatcheries; sites which tested negative for OsHV-1 µvar during the study had significantly lower mortalities than sites which tested positive and mortalities increased with temperature until a peak was reached. There were several differences between the seed stocks from French and non-French hatcheries, including prior OsHV-1 µvar exposure and ploidy. A range of risk factors relating to farm management were also considered, but were not found significant. The relative importance of prior OsHV-1 µvar infection and ploidy will become clearer with ongoing selection towards OsHV-1 µvar resistant oysters. Work is currently underway in Ireland to investigate these factors further, by tracking seed from various hatchery sources which were put to sea in 2012 under similar husbandry and environmental conditions.

Key words: oysters, *Crassostrea gigas,* mortality, Ireland, risk factors

1. Introduction

The Pacific oyster, *Crassostrea gigas*, plays a significant role in the aquaculture industry in Ireland, both in terms of volume and value, with an annual production of over 7,000 metric tonnes (Bord Iascaigh Mhara (BIM) / the Irish Sea Fisheries Board, personal communication). With a current value of €28-30 million per year, *C. gigas* accounts for approximately 20% by volume of overall shellfish produced in Ireland (BIM, personal communication). The main method of cultivation for Pacific oysters used in Ireland is bag and trestle cultivation, which is an off-bottom culture method. This cultivation method allows the oysters to be placed in mesh bags on metal framed structures called trestles in the inter-tidal zone, which allows access to the stock during low tide (Tidwell et al., 2012). Over half of all current licensed aquaculture producers in Ireland are oyster farmers, with *C. gigas* being grown in 44 bays all around the coast. *C. gigas* seed is predominantly sourced from hatcheries / nurseries or harvested wild seed, which is imported mainly from France and to a much smaller extent from the UK and the Channel Islands. The main export market for Pacific oysters is France, although there is also a growing market in Asia.

Since the 1950s, episodes of increased mortality in *C. gigas* have been described globally in all areas of production. In Europe, severe mortality events in cultured Pacific oyster were observed during the summers of 2008 and 2009 (Dégremont et al., 2013). These events have been grouped by life stage into summer mortality in adults, mortality in spat and hatchery-related mortality (European Food Safety Authority, 2010). Mortality in spat and larvae at hatcheries have been associated with detection of ostreid herpesvirus 1 (OsHV-1), a virus also associated with mortality in other farmed bivalves, including the European flat oyster *(Ostrea edulis),* scallop *(Pecten maximus)* and the Manila clam *(Ruditapes philippinarum)* (Renault et al., 2001; Arzul et al., 2002; Batista et al., 2007). In 2000, OsHV-1var, a variant strain of OsHV-1, was identified in French hatcheries (Arzul et al., 2001a,b). Although

OsHV-1var presents several modifications in the C region of the genome, where the most significant modifications in relation to OsHV-1 occur, and a 2.8 kb deletion, OsHV-1 and OsHV-1var are considered representative of a single viral species as the differences between the two genotypes were not great enough to establish a separate viral species (Arzul et al., 2001b). In 2008, the emergence of a third strain was described, OsHV-1 µvar, in association with abnormal mortality in *C. gigas* in France (Segarra et al., 2010; Dégremont et al., 2013). It has since been shown that mortality in *C. gigas* spat can be induced following experimental infection with OsHV-1 µvar (Schikorski et al., 2011). Further, mortality can be induced following horizontal transmission of infection from unselected asymptomatic adult to juvenile *C. gigas* (Dégremont et al., 2013). Since 2009, OsHV-1 µvar has been the predominant herpes virus strain during mortality events (European Food Safety Authority, 2010). It is now believed that the cause of mortality events in *C. gigas* spat and larvae is multifactorial, with OsHV-1 infection (with OsHV-1 µvar now predominating) a necessary but not sufficient cause (Samain and McCombie, 2010). Other suspected risk factors include an increase or a sudden change in the temperature, husbandry practices such as introduction of possibly infected spat, and the movement and mixing of populations and age groups (European Food Safety Authority, 2010; Garcia et al., 2011). The European Food Safety Authority (2010) also noted that no outbreaks had been reported when the water temperature was below 16°C.

In Ireland, mortality events in *C. gigas* have been reported for some years (Malham et al., 2009), but linked, since 2008, to the presence of OsHV-1 µvar (D. Cheslett, personal communication). In that year, reports of mortality in Pacific oysters were received from three oyster producing bays, and the presence of the OsHV-1 µvar was confirmed in all three bays by PCR and sequence analysis of the amplicon (European Food Safety Authority, 2010). In

2009, extensive mortality and the presence of OsHV-1 µvar were reported from 15 oyster production areas, peaking in July with an average batch mortality of 37% and with mortality occurring, on average, over an 18 day period (Peeler et al., 2012). Although few clear associations between mortality and management factors were identified, the age of oysters when first infected with OsHV-1 µvar, the condition of the oysters, temperature, and other environmental factors each appeared important (Peeler et al., 2012). European Union legislation was subsequently introduced to prevent the spread of the virus to unaffected areas in Ireland and the United Kingdom, whilst still allowing trade to continue between infected areas (European Community, 2010), but noting that there was no realistic prospect of eliminating the virus (Peeler et al., 2010). OsHV-1 µvar related mortality has continued in Ireland each summer since the initial detection of OsHV-1 µvar in 2008, both in bays previously infected with this virus and coincident with spread of infection to new bays.

The objectives of the current study were to describe any mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated environmental, husbandry and endogenous oyster factors, thereby providing information which could assist oyster farmers in minimising batch mortality in endemically affected areas.

2. Materials and methods

2.1 Study design and population

A prospective cohort study was conducted during 2010 to 2012, from the time of first batch immersion (03 August 2010), when oysters were two mm in size. The oysters were followed until the date of last batch grading before data analysis, in spring/summer 2012 (12 April

2012), where oysters had been immersed for up to 18 months (subsequently termed the study period). The oyster batch was the unit of interest, and was defined as a variable number of oysters of similar size, originating from one hatchery, placed at a particular site within a bay at one point in time. All 405 batches of farmed oysters that were in the sea in Ireland during 2011 were considered for inclusion in the study. Farms were selected for logistical reasons, such as the accessibility of stock to allow for frequent monitoring and the capacity to monitor individual batches among the stock throughout the season. From the selected farms batches were chosen in order to include batches from different hatcheries, ploidy status and immersion date within each bay.

The number of batches required for the study was estimated, based on a confidence interval of 95% (alpha = 0.05), a power of 80% and assuming a mortality of 43% amongst batches in OsHV-1 positive bays and 12% in OsHV-1 negative bays (based on reported site-level mortality from the 2009 Irish survey (Peeler et al., 2010); data as presented for OsHV-1 positive bays and as estimated for OsHV-1 negative bays). The initial sample size was calculated using the following formula to detect a difference between two proportions (Dohoo et al., 2009) :

$$n = [Z_\alpha \sqrt{(2pq)} - Z_\beta \sqrt{((p_1q_1)+(p_2q_2))}]^2 / (p_1 - p_2)^2$$

Where: $Z_\alpha = 1.96$, the value required for a confidence of 95%, $Z_\beta = -0.84$ the value required for a power of 80%, p_1 = estimate of lower proportion of disease, p_2= estimate of higher proportion of disease, $q_1 = 1-p_1$, $q_2 = 1-p_2$, $p = (p_1 + p_2)/2$ and $q = 1-p$.

The sample size was further adjusted to account for other confounders. Assuming other confounders were not strong confounders the sample size was increased by 15% (Dohoo et al., 2009, page 50). Clustering within farms was accounted for using the following formula:

$$n' = n (1 + \rho (m -1))$$

Where: ρ is the intra-cluster correlation coefficient, assumed to be 0.5 and m is the average number of batches of oysters per farm, assumed to be 3. In total, the aim of the study was to sample around 80 batches.

2.2 Data collection

An initial survey of all study farmers was conducted to gather relevant data on each study batch, and to provide a framework for data collection at batch-level throughout the study period. The survey was administered in a joint effort by BIM regional officers and the Fish Health Unit of the Marine Institute (MI). Each study batch was tracked throughout the study period. To achieve this, BIM Regional Officers made regular visits to survey the batches and collect data on batch identification, risk factors of interest, batch mortality data, splitting, grading and handling frequency. For any batch where mortality occurred, details of the mortality event were recorded (i.e. start and finish date of the mortality episode, estimated percentage mortality in the batch, any predisposing factors). Data were recorded on specifically designed survey forms which were submitted to the MI and entered into a Microsoft Access database. Data collection sheets are available in Appendix A.

Samples of the stock (30 animals from one batch in each bay; Table 1) were tested for the presence of OsHV-1 in the Fish Health Unit laboratory at the Marine Institute. Cell lysis and nucleic acid extraction was carried out using QIAamp DNA Mini Kit (Qiagen) using Qiagen QIAcube, according to the manufacturer's instructions. DNA extracts obtained were subjected to an initial screening process for the presence of OsHV-1 using real-time PCR analysis based on a Sybr Green chemistry with C9/C10 primer set targeting the C region of the genome (Pepin et al., 2008). Confirmatory testing for OsHV-1 µvar was carried using

nested PCR where subsamples of real-time positive PCR products were analysed using nested conventional PCR, using C2/C6 primers (Arzul et al., 2002) in the first round of conventional PCR, and internal primer set F-int/R-int, with a 514bp expected product size in the second round of conventional PCR, using PCR conditions (unpublished, D. Stone, CEFAS, UK).

A total of 55 data loggers, at least one per bay, were deployed to obtain environmental data. These were deployed in May 2011 on a small number of sites, due to availability of equipment, with further deployments made in early June 2011 on the remaining sites. Five different types of data loggers (two large types and three small types) were deployed, each recording a number of different parameters. The smaller data loggers were deployed attached to the bags in the batch of interest, whereas the larger data loggers were deployed alongside the trestles which held the batch of interest. These data loggers were programmed to record environmental parameters every hour for the duration of the deployment, with regular maintenance and calibration on a monthly basis. An illustration of the location of data loggers at Dungarvan bay is shown in Figure 1.

<Figure 1 here>

2.3 Descriptive analysis of temperature at high mortality sites where start date of mortality recorded

A high mortality site was defined as a site where 1 or more batches experienced a cumulative batch-level mortality > 34% (being the upper mortality quartile) during the study period. A low mortality site was defined as a site where no batches experienced a cumulative batch mortality > 34%. At all high mortality sites with available data (reported start date of

mortality event and temperature data during the 30 preceding days), the median temperature within the previous 7 and 30 days prior to the recorded start of mortality were reported. At all low mortality sites, the median temperature during the same time periods as each of the high mortality batches was also reported, for comparison.

At all high mortality sites with available data, a visual assessment of temperature changes was made during the period when mortality events were first recorded. A low-pass filter using Matlab (MathWorks, Natick, MA, USA), was used to remove the tidal, diurnal and other high frequency signals from the temperature time series, in order to assess the overall trend in temperature during the period when mortality occurred, particularly with respect to a 16°C threshold.

2.4 Linear Mixed model analysis

The cumulative batch-level mortality from placement to first grading was the outcome of interest. The independent variables, for consideration during the risk factor analysis, are presented in Table 1.

<Table 1 here>

Due to the highly skewed nature of the mortality data, the natural logarithm was used to transform these to a normal distribution. In addition the appropriate transformation of the outcome was assessed based on the residuals from the final model, comparing the profile likelihood ratio to identify the optimal box-cox transformation (Dohoo et al., 2009).

A mixed linear regression model of cumulative batch-level mortality was developed using SAS version 9.1.3 (SAS Institute Inc., 2003). A univariable screening approach was used, where all variables with $p<0.2$ at the univariable stage became candidates for the multivariable model. The need for a 'farm' random effect was tested within the mixed model, using a likelihood ratio test (Dohoo et al., 2009), to account for clustering of batches within farms. A backward selection procedure was used to eliminate terms from the model based on an F-test ($p>0.05$). The relationship between continuous predictors and the outcome was examined using plots of the continuous predictors against the logarithm of batch mortality. Where no obvious linear or polynomial relationship was observed, continuous predictors were categorised into four groups based on the corresponding quartiles. Variables that were not significant at the univariable screening stage were added to the final model and tested using an F-test ($p<0.05$). In addition, variables that, when combined, represent an underlying effect were tested in combination as described by Cohen et al. (2003) by adding the combined variables to the final model. For example, the variables: 'number of times bags were turned' and 'handling at grading' when combined were considered to represent a 'bag handling' effect. The correlation between covariates was evaluated using a chi-square test between nominal variables and kendall's tau-b assessment of correlation between continuous variables. Plots of studentised residuals from the final model and influence plots were examined to identify lack of fit or any outliers from the final model.

3. Results

3.1 Study population

A total of 80 study batches, from 28 farms, located at 24 sites within 17 infected bays were included in the study (Figure 2). The batches contained between 10 and 4,650 bags of oysters with a median number of 215 bags per batch. The median number of oysters per bag as at the end of May 2011 was 2,000; this varied between 210 to 15,000 oysters per bag between batches. Cumulative batch mortality ranged from 2% to 100%, with a median batch mortality of 16% and an interquartile range of between 10% and 34% (Figures 2 and 3).

<Figure 2 here>

<Figure 3 here>

3.2 Descriptive analysis of temperature at high mortality sites where start date of mortality recorded

There were 14 high mortality sites (mortality >34%), with a total of 20 high mortality batches (out of 52 batches at these sites), and 10 low mortality sites (no batches experienced a cumulative batch mortality > 34%), with a total of 28 batches. Of the high mortality sites, 10 sites had a recorded start date for mortalities, however, three of these sites had missing temperature data (three sites in Ballymacoda, Bannow and Lough Foyle bays). The median temperature within 7 and 30 days of the reported start of mortality at the seven high mortality sites with a recorded start date of mortality and relevant temperature data is shown in Table 2. The median temperatures at the 10 low mortality sites during the same time periods as each of the high mortality sites are also presented in Table 2. The interquartile range for the high mortality sites overlapped the interquartile range for the low mortality sites during the 7 and 30 days time intervals, with the exception of the site at Lough Swilly, indicating that there

272 was little difference in temperature between the low and high mortality sites prior to the start
273 of mortalities.
274
275 <Table 2 here >
276
277 A visual assessment of temperature data during the reported start of mortality at the seven
278 high mortality sites with a recorded start date for mortalities and temperature data prior to the
279 start of mortalities is presented in Appendix B. At four sites (Achill Sound, Dungloe,
280 Trawbreaga Bay and Woodstown Strand), an increase in temperature over 16°C coincided
281 with the time period when mortality events were first reported; at one site (Sherkin), no visual
282 association was observed; and at two sites (Clew Bay, Lough Swilly), a substantial change in
283 temperature, in this case a decline followed by an increase, was coincident with the time
284 period when mortality was first reported.
285
286 3.3 Linear mixed effects regression model
287
288 All predictors with a p-value in the univariable analysis of <0.2 were considered in the initial
289 linear mixed effects regression model (Table 3). The risk factors entered into the initial model
290 were: size, ploidy, OsHV-1 µvar bay status, hatchery and the maximum summer temperature.
291 The remaining significant variables after the backward selection process were: OsHV-1 µvar
292 bay status and hatchery. Variables that were not significant at the univariable stage (p>0.2),
293 and combined variables representing an underlying effect were added to the model, however,
294 none were significant. The studentised residuals were plotted against each of the variables not
295 in the current model using a Loess smooth to identify any potential relationships. The Loess
296 plot against 'maximum summer temperature' suggested a possible quadratic relationship. A

297 quadratic term of the 'maximum summer temperature', after centring the variable to account
298 for correlation between the original value and the quadratic term (Dohoo et al., 2009), was
299 significant when added to the model (Table 4). Residuals from the final model indicated no
300 significant lack of fit and no significant outliers. The appropriate transformation of the
301 outcome variable (cumulative batch-level mortality), based on residuals from the initial
302 model (i.e. containing hatchery and OsHV-1 µvar bay status) and comparing the raw
303 mortality, natural logarithm transformation, arcsine transformation and box-cox
304 transformations were assessed. The natural logarithm transformation was deemed as the most
305 appropriate based on residuals and assessment of box-cox transformations and comparing the
306 profile log-likelihood (Dohoo et al., 2009). The 'farm' random effect was significant
307 (likelihood ratio test: $p = 0.023$) and 38% of the variance was between farms and 62% within
308 farms.
309
310 <Table 3 here >
311
312 A plot of the best unbiased linear estimates of mortality against the 'maximum summer
313 temperature' is shown in Figure 4, indicating an increase in mortality which appears to level
314 off after a temperature peak was reached. The final model also showed that batches imported
315 from French hatcheries 1, 2, 4 and 5 had significantly lower mortalities than batches imported
316 from Non-French hatchery 2. Non-French hatchery 1 mortalities were not significantly
317 different to those from Non-French hatchery 2. Further, batches grown in bays which tested
318 OsHV-1 µvar negative during the study had significantly lower mortalities than batches
319 grown in bays which had tested positive.
320
321 <Table 4 here >

<Figure 4 here>

4. Discussion

In this work, we have sought to identify risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. Increased farmed oyster mortality has been an ongoing concern in Ireland for some years (Malham et al., 2009; Peeler et al., 2012), although there is evidence from the current study of a lower median batch mortality in 2011 (16%) compared with either 2009 (37%) or 2010 (32%). Three risk factors were significantly associated with mortality including the hatchery from which seed was sourced, the presence of OsHV-1 µvar detected in specific bays during 2011 and the maximum temperature observed between June and August 2011, inclusive. Each will be considered in turn.

We noted a strong association between hatchery of origin and mortality, with seed imported from French hatcheries experiencing markedly lower mortality compared with seed imported from non-French hatcheries, when placed in bays which were historically infected with OsHV-1 µvar. As illustrated in Table 3, the median cumulative mortality of batches from French hatcheries varied between 7 and 25%, and from non-French hatcheries between 76 and 86%. There are several differences between these two seed stocks, which may at least partly explain this result. The first relates to prior OsHV-1 µvar exposure. This virus is endemic in France (Pernet et al., 2012). Therefore, prior exposure to this virus, either of the seed itself or of the related broodstock, can be assumed. In contrast, the non-French hatcheries are located in areas outside of Ireland which were not previously infected with OsHV-1 µvar. Prior exposure can lead to latency (Dégremont et al., 2013), a common feature of other herpesvirus infections (Arzul et al., 2002), and OsHV-1 µvar has been identified in

apparently healthy oysters (Arzul et al., 2002; Barbosa-Solomieu et al., 2004; Dégremont et al., 2013). However, prior exposure will not result in a specific immune response, noting that molluscs lack immunological memory, relying entirely on innate immunity to overcome diseases (Gestal et al., 2008; Renault, 2008). Rather, the protective effect of prior exposure is likely genetic (Dégremont et al., 2007; Sauvage et al., 2009; Huvet et al., 2010), with oysters surviving a mortality event being naturally selected for resistance to disease (Dégremont et al., 2010; Pernet et al., 2012). In recent years, considerable progress has been made in France towards selection for OsHV-1 µvar resistant oysters, particularly in the context of summer mortality in adults (Dégremont et al., 2010; Dégremont et al., 2013). The second difference between the seed stocks relates to ploidy: a substantial proportion of the French-derived batches in this study were triploid (88%), whereas most of the non-French-derived batches were diploid (75%). In the current study, there was a univariable association between ploidy and cumulative batch mortality (with diploid stock being at greater risk, Table 3), however, ploidy was not retained in the final multivariable model (Table 4). This result is at odds with an earlier Irish study, where triploid oysters were at greater mortality risk (Peeler et al., 2012), but consistent with studies on adults from France, prior to 2006 at least (Gagnaire et al., 2006; Samain, 2011), which found triploid oysters were more resistant to summer mortality. This latter effect varied by season (Pernet et al., 2012), possibly due to seasonal differences in reproductive effort and immunological parameters. Given the data available, it is not possible to disentangle the relative importance of ploidy and prior OsHV-1 µvar exposure in the current study. The association with ploidy could be a consequence of confounding, noting that most of the French seed was triploid and most of the seed imported from non-French hatcheries was diploid. The observed effect could be due to innate resistance among stock from French hatcheries to OsHV-1 µvar infection.

371 There was also some variation in the mortality of batches from different French hatcheries,
372 with median mortality varying between 7% and 25% (Table 3), although the sample size was
373 too small to conduct a formal analysis as to whether these differences in mortality were
374 significant. In these hatcheries, diploid females are produced locally, whereas the male
375 tetraploids are owned by the French government but moved from one hatchery to another for
376 the purposes of fertilization. This may reflect a degree of genetic selection in specific
377 hatcheries over the period since 2007/08 when the disease first appeared in France. A genetic
378 component, leading to hatchery differences, has previously been noted for summer mortality
379 in *C. gigas* adult oysters (Dégremont et al., 2007; Sauvage et al., 2009; Huvet et al., 2010).
380
381 In this study, the OsHV-1 µvar status of the bay was significantly associated with mortality,
382 (median mortality of 20% and 10% among bays where OsHV-1 µvar was detected, or not,
383 during 2011, respectively, Table 3). In this context, bay status is best interpreted in terms of
384 viral load during 2011, as opposed to either presence or absence, noting that we used a
385 sampling strategy to provide 95% confidence that virus would be detected, if present at a
386 specified design prevalence of 10% or greater (Table 1). Each of the 17 study bays can be
387 considered endemically infected: OsHV-1 µvar had been detected in each, either during 2011
388 or previously, and the virus is known to persist in adult oysters following primary infection
389 (Lipart and Renault, 2002; Dégremont et al., 2013). These observations are consistent with
390 earlier work, highlighting increased mortality risk with increasing quantities of OsHV-1 µvar
391 DNA, but frequently in the context of summer mortality in adults (Pepin et al., 2008; Sauvage
392 et al., 2009; Schikorski et al., 2011; Garcia et al., 2011).
393
394 We found a quadratic relationship between the natural logarithm of the cumulative mortality
395 and the maximum temperature observed between June and August 2011, noting that the

maximum temperature observed in this study may have occurred as a one day event rather than a sustained rise in temperature. In addition, for the seven high mortality sites at which mortality date and temperature data were available, temperature had exceeded 16°C in the week prior to mortalities at five of the sites. At the other 2 sites, temperatures had either exceeded 16°C in the previous month (Sherkin) or were around 16°C in the previous week (Woodstown Strand) (Appendix B). However, there was little difference in the average temperature in the time prior to the mortalities when compared to sites with low mortalities (Table 2). A number of studies have highlighted the multifactorial nature of mortality events in oyster spat and larvae, with the European Food Safety Authority (EFSA, 2010) stating that climatic factors were unlikely to be a sufficient cause for summer mortalities. However, this publication also noted the absence of mortality outbreaks when the water temperature had been below 16°C. Several authors have highlighted the potential role of increased water temperature in oyster mortality events. During 1998 to 2006 in France, OsHV-1 was often detected when temperatures increased quickly, but was no longer detected once temperatures were stable even if they remained high (Garcia et al., 2011). Further, a temperature increase may lead to a re-activation of latent infection (Sauvage et al., 2009). According to data collected from M1-M5 Databuoys deployed around the Irish coast, water temperatures were significantly lower in 2011 than in previous years (Marine Institute, http://www.marine.ie/home/publicationsdata/data/buoys/). Although several authors have highlighted the role of farm management (Samain and McCombie, 2010; European Food Safety Authority, 2010), no relevant variables were significant in the current multivariable modelling.

There are a number of limitations to the current study which we note here. We were reliant on farmer recall to determine the start date of mortalities. This proved problematic, however,

both in terms of data validity and completeness. Further, the precision of these estimates is influenced by the frequency with which farmers check their stock. In most cases, due to tidal patterns which lead to site inaccessibility, stock can only be checked at best every two weeks, and in some cases stock may not be checked for up to a month. Similar challenges have been described previously among French oyster farmers, where yearly variation in reporting sensitivity has been observed. Increases in reporting sensitivity were found to occur concurrent with outbreak occurrence and with implementation of financial incentives to encourage farmer reporting of mortality events (Lupo et al., 2012). The cumulative mortality estimate for each batch in the study was based on the outcome of grading for each batch in early spring 2012. Some farmers with several study batches found it difficult to obtain grading data for individual batches as grading would usually be carried out on a stock basis rather than a batch basis. We also faced some gaps in the environmental data, noting that recordings were not taken during the same time period across all sites. In addition, although data were available on a range of environmental parameters, only the temperature data proved suitable for subsequent analysis. Extra environmental data were only recorded at a limited number of sites due to cost and availability of monitoring equipment. There were some issues with tracking batches once they had been split. Several batches were split during the season, resulting in new stocking densities with new bag mesh sizes, and several new locations for the split batch. This proved problematic when tracking split batches through the season, as once the original batch was split, all subsequent batches had a separate set of data, and were essentially a new batch. Initial sample size calculations were based on the difference in the proportion of mortality in two groups. However, for the analysis linear regression with cumulative mortality as the outcome was used. If sample size calculations had been based on the difference between two mortality rates as described by Lwanga and Lemeshow (1990) there would have been very little difference in the estimated sample size. Finally, the farms

and study batches were not chosen at random, primarily for logistical reasons. It is possible that these samples are not representative of the broader oyster farming population in Ireland.

The study provides some insights into mortality events affecting farmed Pacific oyster production in Ireland. Batch mortality was lower in 2011, compared with earlier years, in association with lower viral loads. Mortality was significantly associated with hatchery source, for reasons that are currently unclear, and with water temperature. Further work is needed to elucidate the basis for this effect. The relative importance of prior OsHV-1 µvar infection and ploidy will become clearer with ongoing selection towards OsHV-1 µvar resistant oysters. Work is currently underway in Ireland to investigate these factors further, by tracking seed from various hatchery sources which were put to sea in 2012 under similar husbandry and environmental conditions.

Acknowledgements

We gratefully acknowledge the assistance of participating oyster farmers, data collection by Bord Iascaigh Mhara inspectors and Marine Institute staff and assistance from Dan Collins (UCD Centre for Veterinary Epidemiology and Risk Analysis) with mapping.

Conflicts of interest

The authors do not have any conflict of interest.

Appendices A and B. Supplementary data

Supplementary data associated with this article can be found, in the online version.

471 **References**

472

473 Arzul, I., Nicolas, J.L., Davison, A.J., Renault, T., 2001a. French Scallops: A New Host for
474 Ostreid Herpesvirus-1. Virology 290, 342-349.

475

476 Arzul, I., Renault, T., Lipart, C., Davison, A.J., 2001b. Evidence for interspecies transmission
477 of oyster herpesvirus in marine bivalves. J. Gen. Virol. 82, 865-870.

478

479 Arzul, I., Renault, T., Thébault, A., Gérard, A., 2002. Detection of oyster herpesvirus DNA
480 and proteins in asymptomatic *Crassostrea gigas* adults. Virus Res. 84, 151-160.

481

482 Barbosa-Solomieu, V., Miossec, L., Vázquez-Juárez, R., Ascencio-Valle, F., Renault, T.,
483 2004. Diagnosis of ostreid herpesvirus 1 in fixed paraffin-embedded archival samples using
484 PCR and in situ hybridisation. J. Virol. Methods 119, 65-72.

485

486 Batista, F.M., Arzul, I., Pepin, JF., Ruano, F., Friedman, C.S., Boudry, P., Renault, T., 2007.
487 Detection of ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review. J.
488 Virol. Methods 139, 1-11.

489

490 Cohen, J., Cohen, P., West, S.G., Aiken, L.S., 2003. Applied multiple regression/correlation
491 analysis for the behavioural sciences. Third Edition. Routledge, New York, USA.

492

493 Dégremont, L., Boudry, P., Ropert, M., Samain, JF., Bédier, E., Soletchnik, P., 2010. Effects
494 of age and environment on survival of summer mortality by two selected groups of the
495 Pacific oyster *Crassostrea gigas*. Aquaculture 299, 44–50.

Dégremont, L., D., Guyader, T., Tourbiez, D., Pepin, JF., 2013. Is horizontal transmission of the ostreid herpesvirus OsHV-1 in *Crassostrea gigas* affected by unselected or selected survival status in adults to juveniles? Aquaculture [doi: 10.1016/j.aquaculture.2013.05.025]

Dohoo, I., Martin, W., Stryhm, H., 2009. Veterinary Epidemiologic Research 2nd Edition. VER Inc., Charlottetown, PEI, Canada.

European Community, 2010. 2010/221/EU: Commission decision of 15 April 2010 approving national measures for limiting the impact of certain diseases in aquaculture animals and wild aquatic animals in accordance with Article 43 of Council Directive 2006/88/EC. Official Journal of the European Communities L98:7-11. 20 April 2010 [including subsequent amendments].

European Food Safety Authority, 2010. Scientific opinion on the increased mortality events in Pacific oysters, *Crassostrea gigas.* EFSA J. 8, 1894.

Gagnaire, B., Soletchnik, P., Madec, P., Geairon, P., Le Moine, O., Renault, T., 2006. Diploid and triploid Pacific oysters*, Crassostrea gigas* (Thunberg), reared at two heights above sediment in Marennes-Oleron Basin, France: difference in mortality, sexual maturation and hemocyte parameters. Aquaculture 254, 606–616.

Garcia, C., Thébault, A., Dégremont, L., Arzul, I., Miossec, L., Robert, M., Chollet, B., François, C., Joly, J.P., Ferrand, S., Kerdudou, N., Renault, T., 2011. Ostreid herpesvirus 1

520 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and
521 2006. Vet. Res. 42, 73.
522
523 Gestal, C., Roch, P., Renault, T., Pallavicini, A., Paillard, C., Novoa, B., Oubella, R., Venier,
524 P., Figueras, A., 2008. Study of diseases and the immune system of bivalves using molecular
525 biology and genomics. Rev. Fish. Sci. 16, 133-156.
526
527 Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., Boudry, P., 2010. Reproductive
528 effort of Pacific oysters: A trait associated with susceptibility to summer mortality.
529 Aquaculture 304, 95–99.
530
531 Lipart, C., Renault, T., 2002. Herpes-like virus detection in infected *Crassostrea gigas* spat
532 using DIG-labelled probes. J. Virol. Methods 101, 1-10.
533
534 Lupo, C., Osta, A.A., Mandard Y.V., Peroz C., Arzul, I., Francois, C., Garcia, C., Renault, T.,
535 2012. Sensitivity of mortality reporting by the French oyster farmers. 13th Conference of the
536 International Symposium on Veterinary Epidemiology and Economics (ISVEE XIII), 20-24
537 August 2012, Maastricht, the Netherlands. http://archimer.ifremer.fr/doc/00102/21343/
538
539 Lwanga, S.K., Lemeshow, S., 1991. Sample size determination in health studies. A practical
540 manual. World Health Organization, Geneva, Switzerland.
541
542 Malham, S.K., Cotter, E., O'Keeffe, S., Lynch, S., Culloty, S.C., King, J.W., Latchford, J.W.,
543 Beaumont, A.R., 2009. Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the

Irish Sea: The influence of temperature and nutrients on health and survival. Aquaculture 287, 128–138.

Peeler, E.J., Reese, A., Thrush, M.A., 2010. Report on investigation of oyster herpes virus infection and oyster mortality in the Republic of Ireland in 2009 – a questionnaire survey. Centre for Environment, Fisheries & Aquaculture Science (Cefas), Weymouth, UK. July 2010.

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, 136-143.

Pepin, 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, 269-76.

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Pepin, J.F., Keck, N., 2012. Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquacult. Environ. Interact. 2, 215–237.

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.C., 2008. Genomics and mollusc pathogens: trends and perspective. J. Vet. Clin. Sci. 1, 36–46.

Samain, J.F., McCombie, H. (eds), 2010. Summer mortality of Pacific oyster *Crassostrea gigas*. The Morest project. Éditions Quæ.

Samain, J.F., 2011. Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster *Crassostrea gigas* to summer mortality. Aquat. Living Resour. 24, 227–236.

Sauvage, C., Pepin, 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.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., Pepin, JF., 2011. Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Vet. Res. 42, 27.

Segarra, A., Pepin, JF., 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.

Tidwell, J. H., 2012. Aquaculture Production Systems. Wiley-Blackwell, Oxford, UK.

Tables:

Table 1. Independent variables considered in the study

Table 2. The median and interquartile range of temperature recorded within 7 and 30 days of the reported start of mortalities at seven high mortality sites (those with 1 or more batches with a cumulative mortality > 34%) during summer 2011 in Ireland. The median and interquartile range of temperatures at the ten low mortality sites (those sites without any high mortality batches) during the same time periods is included for comparison.

Table 3. The median cumulative mortality of oyster batches in Ireland in 2011, along with the interquartile range by categorical risk factors that met the criteria for inclusion in the multivariable model ($p<0.2$).

Table 4. Linear mixed regression model of the log of batch mortality among 80 batches of oysters in Ireland in 2011

Figures:

Figure 1. The location of study batches and environmental data loggers (Hydrolab DS5 Multiparameter Datasonde loggers; _CTD loggers (DGS – Dungarvan South and DGN – Dungarvan North); RCM9 loggers) in Dungarvan Bay during 2011-12

Figure 2. Location of the 80 study batches and 17 bays, and the cumulative batch mortality (average, range) in each bay, of farmed *C. gigas* in Ireland during summer 2011

618 Figure 3. Distribution of the cumulative mortality (%) among the 80 study batches in Ireland
619 during summer 2011
620
621 Figure 4. Predicted best linear unbiased estimator of the cumulative mortality from the
622 multivariable mixed linear model plotted against the maximum summer temperature, for each
623 hatchery in OsHV-1 μvar positive bays
624

624 Table 1. Independent variables considered in the study

625

Independent variable	Explanation
Time in water	Measured in days from date of immersion until date of final batch grading. Also grouped into time when placed in water *(2010, Spring 2011 and Summer 2011)*
Age placed in water	*2-3, 4-7, >7 (months)*
Ploidy	*Diploid, triploid*
Bay	17 bays
Position on shore	*Low, mid, high*
OsHV-1 µvar bay status[a]	OsHV-1 µvar detection in 2011 *(positive/negative)*
Bag turning	Number of times each bag turned between May to August 2011
Transport	*Air, sea and road, road*
Journey duration	*≤24 hrs, >24 and <36hrs, ≥36hrs and <42hrs, ≥42 hrs*
Bag mesh size	*<4, 4 and >4 (mm)*
Hatchery	The 7 hatcheries from which the oysters originated
Handling at grading	*Manual, mechanical, both*
Sea bed type	*Gravel, gravel/sand, mud, mud/sand, sand*
Average stocking density	*Average no. of oysters per bag.* If bags were split, oyster density was as estimated at the end of May 2011
Bag split	Whether bags were split during the study into smaller number of oysters *(Yes/No)*
Maximum summer temperature	Maximum temperature reached from the start of June until the end of August 2011, measured at 23 sites with between 1 and 8 batches

at each site.

a. Sampling was carried out on a bay basis (i.e. 30 animals from one batch in each bay was sampled for the presence of OsHV-1, the sample size was based on detecting a design prevalence of 10% or greater with 95% confidence), however, the batch sampled may not have been part of this study. A bay was considered positive if at least one batch was infected.

630 Table 2. The median and interquartile range of temperature recorded within 7 and 30 days of the reported start of mortalities at seven high
631 mortality sites (those with 1 or more batches with a cumulative mortality > 34%) during summer 2011 in Ireland. The median and interquartile
632 range of temperatures at the ten low mortality sites (those sites without any high mortality batches) during the same time periods is included for
633 comparison

		At each high mortality site			Among the ten low mortality sites[a]		
Bay[a]	Reported start of mortality	Temperature			Temperature		
		Median	interquartile range		Median	interquartile range	
			Q1	Q3		Q1	Q3
Within 7 days of start of mortalities							
Achill Sound	14-Jul-11	15.9	14.9	16.7	15.3	14.5	16.5
Clew Bay	20-Jul-11	14.8	13.9	15.8	15.0	13.7	16.7
Dungloe	03-Jul-11	15.7	15.3	16.9	14.4	13.5	15.7
Lough Swilly	22-Jul-11	18.8	15.5	20.0	14.4	13.4	15.4
Sherkin	11-Aug-11	14.8	14.5	15.1	15.9	15.4	16.3
Trawbreaga Bay	26-Jul-11	15.2	14.4	16.9	14.9	14.1	16.1
Woodstown Strand	26-Jul-11	14.8	14.4	15.6	14.9	14.1	16.1

Within 30 days of start of mortalities							
Achill Sound	14-Jul-11	17.0	15.6	18.8	19.2	18.0	19.8
Clew Bay	20-Jul-11	15.0	14.2	15.8	15.3	14.0	18.5
Dungloe	03-Jul-11	15.5	15.0	16.2	19.3	18.4	19.9
Lough Swilly	22-Jul-11	20.5	17.4	22.9	14.8	13.7	16.0
Sherkin	11-Aug-11	14.5	13.7	14.9	15.8	14.8	16.5
Trawbreaga Bay	26-Jul-11	15.2	14.3	16.3	15.0	14.0	16.3
Woodstown Strand	26-Jul-11	15.1	14.6	15.7	15.0	14.0	16.3

634 a. The bay may also contain other sites

635 Table 3. The median cumulative mortality of oyster batches in Ireland in 2011, along with the
636 interquartile range by categorical risk factors that met the criteria for inclusion in the
637 multivariable model (p<0.2)

Risk factor		No. of batches	Cumulative batch mortality			P-value[a]
			Q1	Median	Q3	
Size (mm)	2/3	3	25	39	45	0.083
	4/6	66	9	14	31	
	7 +	11	20	34	57	
Ploidy	Diploid	17	10	33	82	0.059
	Triploid	63	8.7	15	32	
OsHV-1 µvar bay	Negative	13	8	10	12	0.199
status	Positive	67	10	20	35	
Hatchery	French hatchery 1	43	9	13	23	0.005
	French hatchery 2	8	9	19	41	
	French hatchery 3	1	25	25	25	
	French hatchery 4	1	7	7	7	
	French hatchery 5	15	8	15	39	
	Non-French hatchery 1	7	12	86	100	
	Non-French hatchery 2	5	34	76	82	

638 a. P-value is based on a univariable linear mixed model using log of the final mortality as the
639 outcome variable and 'farm' as a random effect

640 Table 4. Linear mixed regression model of the log of batch mortality among 80 batches of
641 oysters in Ireland in 2011

Variable	Categories	Estimate	Standard error	P value
Intercept		4.57	0.47	<0.001
Hatchery	French hatchery 1	-1.73	0.46	<0.001
	French hatchery 2	-1.37	0.52	0.010
	French hatchery 3	-1.20	0.84	0.157
	French hatchery 4	-1.97	0.87	0.026
	French hatchery 5	-1.33	0.50	0.011
	Non-French hatchery 1	-0.01	0.53	0.982
	Non-French hatchery 2	0.00	.	.
OsHV-1 µvar bay status	Negative	-0.85	0.37	0.035
	Positive	0.00	.	.
Max. summer temp		0.05	0.03	0.116
Max. summer temp^2		-0.01	0.005	0.040
Random effects		Variance estimate	Standard error	
Farm		0.270	0.160	
Residual		0.443	0.095	

642

643

Figure 1. The location of study batches and environmental data loggers (Hydrolab DS5 Multiparameter Datasonde loggers, _CTD loggers (DGS – Dungarvan South & DGN – Dungarvan North); RCM9 loggers) in Dungarvan Bay during 2011-12

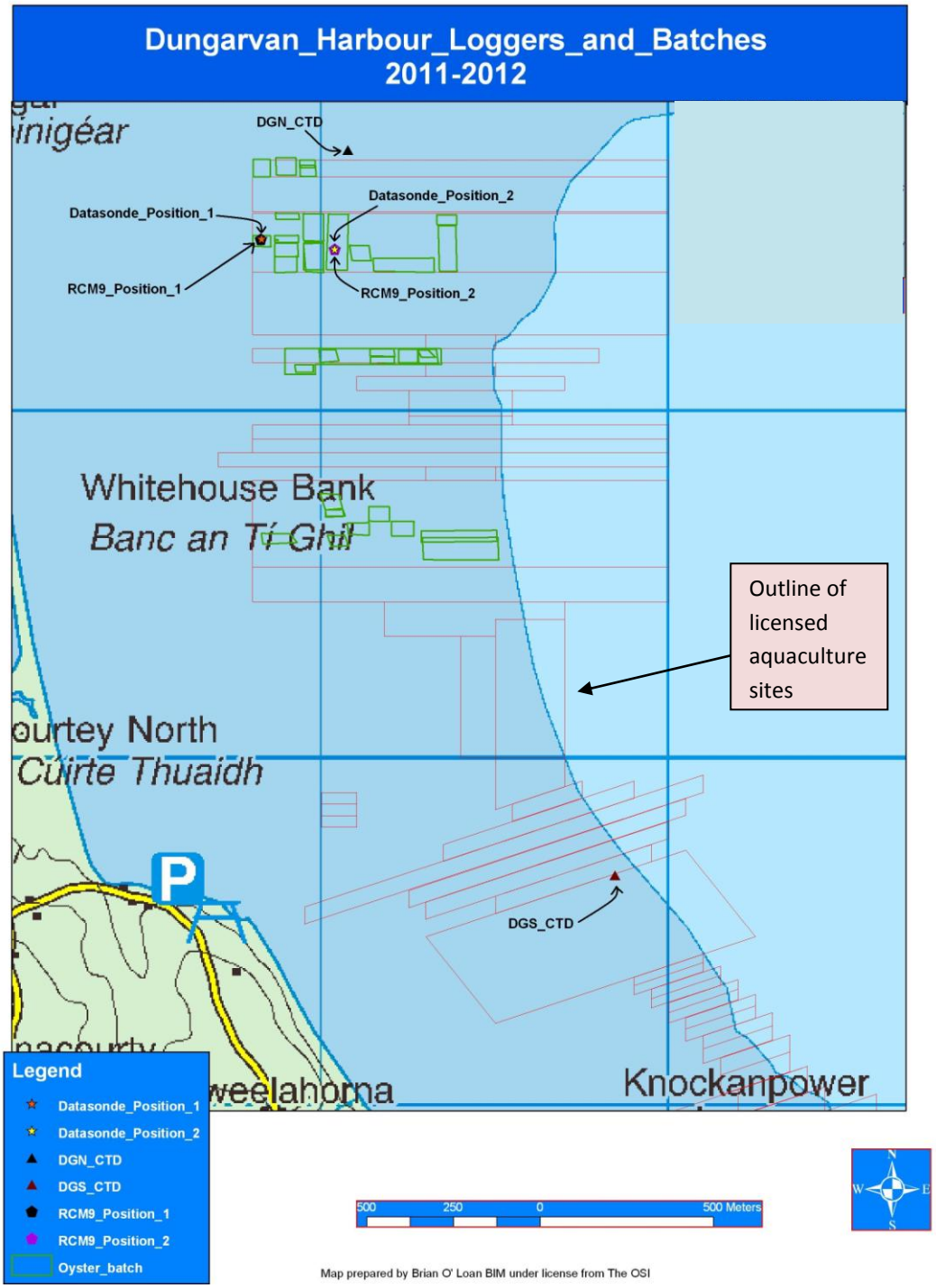

Figure 2. Location of the 80 study batches and 17 bays, and the cumulative batch mortality (average, range) in each bay, of farmed *Crassostrea gigas* in Ireland during summer 2011

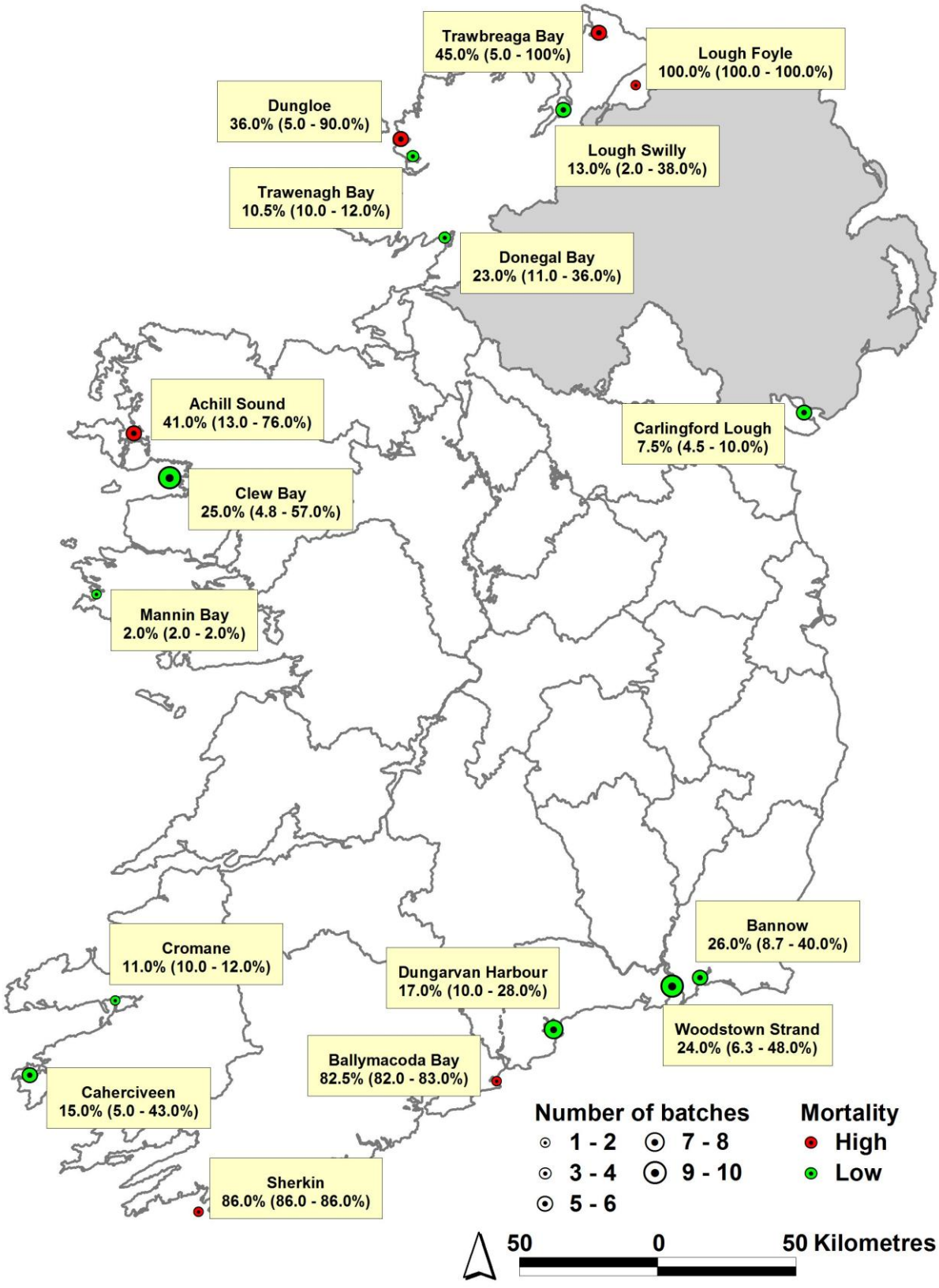

Figure 3. Distribution of the cumulative mortality (%) among the 80 study batches in Ireland during summer 2011

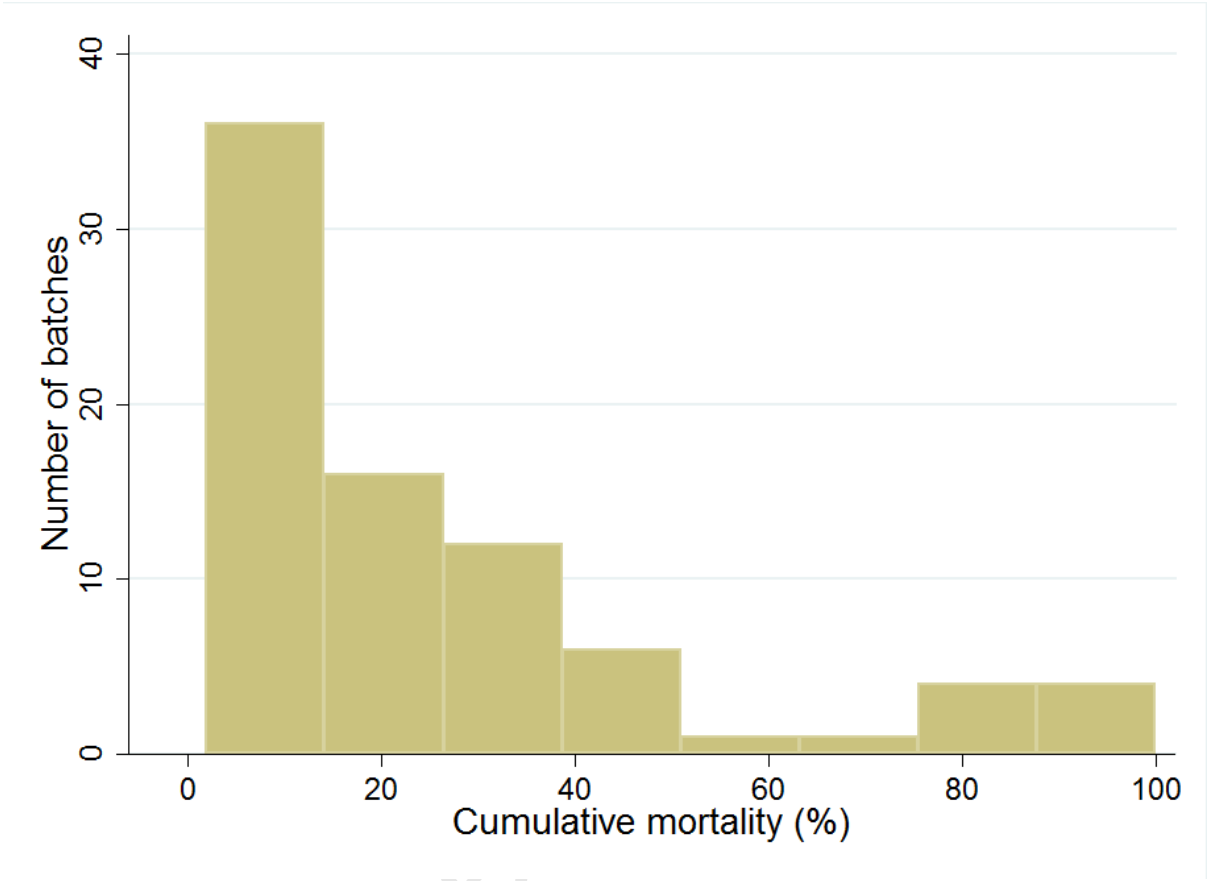

Figure 4. Predicted best linear unbiased estimator of the cumulative mortality from the multivariable mixed linear model plotted against the maximum summer temperature, for each hatchery in OsHV-1 µvar positive bays