Seasonal variation in expression of immune associated genes in the oyster *Ostrea edulis* infected with the parasite *Bonamia ostreae* challenge efforts for marker-assisted breeding for resistance.

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Introduction

The inability of marine invertebrates to develop a competent immune response to specific pathogens has led to mortality events in populations of commercially valuable species such as blue mussels (*Mytilus edulis*) and European flat oyster (*Ostrea edulis*) (Mydlarz, Jones and Harvell, 2006). *Bonamia ostreae* is one such pathogen that was first reported in Europe in 1979 (Pichot *et al.*, 1979), and has since contributed to an > 85 % decline in *O. edulis* worldwide (Beck *et al.*, 2011; Grabowski *et al.*, 2012). *B. ostreae* is a haplosporidian parasite that infects the haemocytes of *O. edulis* and manifests as the disease bonamiosis, which ultimately leads to death (Robert *et al.*, 1991; da Silva, Fuentes and Villalba, 2005; Laing, Walker and Areal, 2005; Lallias *et al.*, 2010). Evidence of a naturally developing resistance amongst *Bonamia*-exposed *O. edulis* has led contemporary attempts to identify markers of *Bonamia*-resistance in wild and commercial stocks of *O. edulis* (Morga *et al.*, 2012; Ronza *et al.*, 2018). Morga *et al.*, (2012) identified seven expressed sequence tags (ESTs) as potential markers of *Bonamia*-reistance: Inhibitor of apoptosis (OeIAP), fas-ligand (OeFAS), cathepsin B (Cathep), extracellular superoxide dismutase (OeEc-SOD), ferritin (Oefer), C1q (OeC1q), and Cyclophylin B (Oepepti). Established reference markers such as these would benefit restoration programmes in the selection of appropriate broodstock for relaying, but further evidence to support this relatively new concept is required.

Oyster populations

B. ostreae is now present in eight European countries with natural and commercial populations of *O. edulis* (Engelsma *et al.*, 2014). Laing *et al.* (2014) found oysters infected with *B. ostreae* at 100 of 145 monitored sites in England and Wales over the course of 30 years. The Solent oyster fishery in the south of England historically produced high numbers of *O. edulis* for consumption each year, landing 650-850 tonnes in 1979-1980, contributing to the huge demand from restaurants on the coast and in London (Helmer *et al.*, 2019). A combination of factors including overexploitation, extreme weather events and the accidental introduction of *B. ostreae* to the south coast of England in 1982 have contributed to the rapid decline in population abundance resulting in repeated closures of the fishery (Hudson and Hill, 1991; Laing *et al.*, 2014). The Rossmore oyster company in Loch Ryan, Scotland, is an *O. edulis* fishery currently naïve to *B. ostreae* (Fariñas-Franco *et al.*, 2018). Privately owned and managed by one oyster grower, this fishery produces up to 10 tonnes of *Bonamia*-free oysters for commercial and restoration use per year (Donnan, 2007). These two working fisheries with different disease status presented a good opportunity to compare the profile of genes identified as *Bonamia*-resistant and the metabolome of oysters from *Bonamia*-naïve (Loch Ryan) and *Bonamia*-exposed (Solent) areas.

Genes

A number of genes associated with oyster immunity have been identified as potential markers of *Bonamia*-resistance in oysters and have inspired debate into the possibility of pathogen recognition mechanisms in invertebrates (Morga *et al.*, 2012; Ronza *et al.*, 2018). Morga *et al.* (2012) observed higher expression of OeIAP, OeFAS and OeEcSOD in *Bonamia*-resistant oysters in comparison to wild oysters. However, Morga *et al.* (2012) only studied populations at one time point and did not explore any temporal component of gene expression between different populations. As such, Morga *et al.*, (2012) only provides a snapshot into gene expression at one event, overlooking any seasonal variation. Therefore, the imperative was to test the findings of Morga *et al.* (2012) in different populations and through a seasonal cycle throughout the year 2018, which has not been attempted before.

This study sought to test six gene markers of resistance proposed by Morga *et al.* (2012) for two different populations through the course of one growth season. Expression of OeIAP, OeFAS, OeEcSOD, OeC1q, Ubiq and ACT were compared between the *Bonamia*-naïve oysters from Loch Ryan Scotland, with *Bonamia*-exposed oysters from the Solent England.

Materials and methods

Oyster husbandry

Oysters were acquired in from Scotland (Rossmore Oysters, Loch Ryan) and England (Portsmouth & Langstone harbours in the Solent). Oysters were collected from the seabed using oyster dredges and sent to the National Oceanography Centre via post in either wooden or polystyrene crates and were out of water for < 48 h. Individuals were then gently scrubbed to remove unwanted epifauna and tagged with numbered fragments of elastic band glued onto their shell. Individuals from Loch Ryan were kept tanks with 2 µm sand filtered seawater from the Solent maintained at their local temperature at the time of harvest (table 1).

Table 1. Dates of oyster arrival to the National Oceanography Centre and tank seawater temperature at which they were held.

	Loch Ryan	***Solent***
Winter	Batch F 28/02/2018	Batch D 21/11/2017
	8.7 °C ± 0.15	7.4 °C ± 0.17
Spring	Batch J 08/05/2018	Batch H 18/04/2018
	10.6 °C ± 0.07	11.6 °C ± 0.41
Summer	Batch K 06/09/2018	Batch M 24/09/2018
	18.9 °C ± 0.92	17.7 °C ± 0.05
Autumn	Batch N 05/11/2018	Batch P 14/11/2018
	10.7 °C ± 0.32	12.4 °C ± 0.05

Oysters from the Solent were kept in an outside tank with a flow through system with water directly from the Southampton Water. Oyster tanks were cleaned every three days changing no more than 30% of new sea water to avoid shock. Oyster tissue was taken within 1 week of arrival.

Feeding

Oysters were likely fed at least twice before sacrifice with a mixed diet of algae; *Isochrysis galabana*, *Tetraselmis suecca* and *Phaeodactylum tricornutum* were added to each tank at a concentration of 40 000 cells ml^{-1}.

Tissue samples

The right valve of each oyster was removed by severance of the adductor muscle from the shell using an oyster knife and surgical blade to minimise damage to the soft tissue. Tissue samples were taken from the hepatopancreas and gill regions (figure 1), flash frozen in liquid nitrogen and held at -20 ˚C and -80 ˚C for later DNA and RNA analysis respectively.

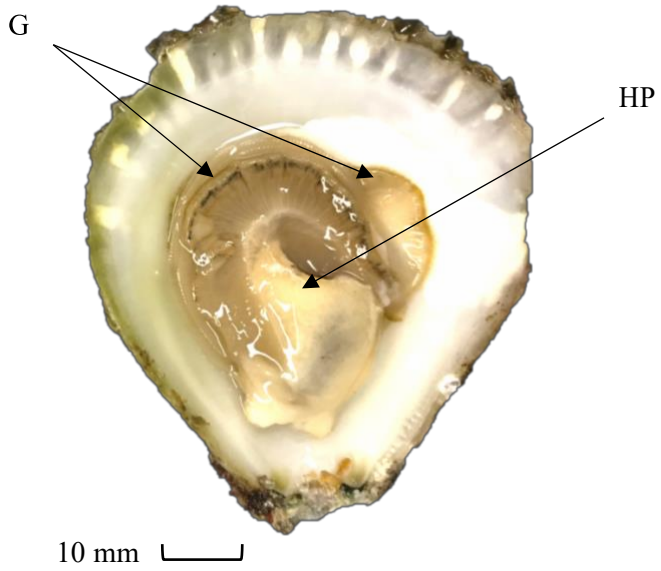


Figure 1. Oyster with location of tissues taken for analysis: hepatopancreas (HP), and gill (G) tissue taken for molecular analysis.

Molecular analysis

Parasite abundance

Bonamia ostreae infection was identified with the use of polymerase chain reaction (PCR) followed by gel electrophoresis. Deoxyribonucleic acid (DNA) was extracted from gill tissues using QIAGEN™ DNeasy Blood and Tissue Kit. Where present, parasite DNA was then amplified using PCR with 30 cycles heating to 95 ºC (1 minute), cooling to 59 ºC (1 min), and warming to 72 ºC (1 min). The primers used to target *B. ostreae* were *BOSTRE* f: TTA CGT CCC TGC CCT TTG TA, *BOSTRE* r: TCG CGG TTG AAT TTT ATC

GT, as developed by Ramilo *et al.* (2013). Amplified DNA fragments from *B. ostreae* were size fractionated by gel electrophoresis and identified after staining with an intercalating DNA binding dye ethidium bromide before visualisation on a transilluminator.

Expression of genes associated with *Bonamia* resistance

Total Ribonucleic acid (RNA) was extracted from gill tissues using a manual protocol involving TriReagent. MIQE compliant quantitative PCR (qPCR) was used to quantify the expression of 6 genes (OeIAP, OeEc-SOD, OeC1q, OeFAS, Ubiq and ACT) associated with *Bonamia* resistance as described by Morga *et al.* (2012) (Table 2).

Four reference genes were used for the qPCR procedure to comply with MIQE standard requirements; glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), Elongation factor 1 alpha (Ef1-α), 60S ribosomal protein L5 (L5), established for *Ostrea edulis* assays by Morga *et al.* (2010), and Cathepsin B (Cathep) that demonstrated a stable level of expression across our samples. The qPCR assay was optimised by first manipulating the primer concentration of each gene to find the best combination for an early and clear cycle threshold (ct) value. The chosen concentration was then taken forward to create a standard curve with 10-fold dilution of cDNA to measure the efficiency of each gene assay.

Table 2. The 10 genes and respective sequences and their melting temperature (T_m). Reference genes marked with asterisk '*'

Gene	**Sequence (5'-3')**	**T_m (°C)**	**Sequence origin**
GAPDH *	F: TCCCGCTAGCATTCCTTG	56.0	(Morga *et al.*, 2010)
	R: TTGGCGCCTCCTTTCATA	53.7	
Ef1-α *	F: GTCGCTCACAGAAGCTGTACC	58.5	(Morga *et al.*, 2010)
	R: CCAGGGTGGTTCAAGATGAT	58.3	
L5 *	F: TCAGTGCAGAGGTTCACAGG	59.4	(Morga *et al.*, 2010)
	R: TAGCAGCATGGCACTTTTTG	55.3	
Cathep *	F: CTGCACTGATCTGGGACTCA	59.4	(Morga *et al.*, 2012)
	R: ATTACYGGCTGGTGGCAAAC	58.3	
Ubiq	F: ACCAAATGAAGCGTGGATTC	55.3	(Morga *et al.*, 2010)
	R: TGAGGTCGAACCATCAGACA	57.3	
ACT	F: ACCAGTTGTACGACCGGAAG	58.6	(Morga *et al.*, 2010)
	R: CACGGTATCGTCACCAACTG	58.2	
OeIAP	F: TCGCGGTTGAATTTTATCGT	59.4	(Morga *et al.*, 2012)

	R: CACCACTCTCCTCCATGTCA	59.4	
OeEc-SOD	F: GAGGAGGAAGAGGACCATCC	61.4	(Morga *et al.*, 2012)
	R: ATTTTCCTCCGCTTTGTGTG	55.3	
OeC1q	F: CAGTCCCTCAGAGCCTGTTC	61.4	(Morga *et al.*, 2012)
	R: ACAGGTATACGCCGGTTTTG	57.3	
OeFAS	F: TTTGGGCAGTGGTGTAAGTG	57.3	(Morga *et al.*, 2012)
	R: TAGCCCTGTTTCTCCACCAG	59.4	

Statistical analysis

All statistical analyses were conducted in the R environment (R Core Team, 2018) with packages ggbiplot, lsmeans, ggplot2 and devtools (Wickham, 2010; Vu, 2011; Wickham, Hester and Chang, 2014; Lenth, 2016). A generalised linear model (GLM) was fitted to the data where possible.

Results

Parasite abundance

B. ostreae was found present in 14 % of the oysters (n = 64) and only within the Solent population.

Expression of genes associated with Bonamia-resistance

The expression of 6 genes (OeIAP, OeEc-SOD, OeC1q, OeFAS, Ubiq, and ACT,) was compared between oyster origin (Loch Ryan (*Bonamia*-naïve) and the Solent (*Bonamia*-exposed)), season (winter, spring, summer and autumn), and between oysters with and without a *B. ostreae* infection

To compare gene expression between the two populations within each season, and between oysters with and without a *B. ostreae* infection, each gene was analysed individually.

Effect of *Bonamia ostreae*

The interaction between *B. ostreae* infection and season explained statistically significant amounts of variance in expression of OeEcSOD (GLM, $F_2 = 3.580$, $P = 0.034$), OeC1q (GLM, $F_2 = 3.533$, $P = 0.036$), and OeFAS (GLM, $F_2 = 9.247$, $P = 0.000$). This suggests that the difference in expression of these three genes each season depended on whether the oyster had a *B. ostreae* infection.

The presence of *B. ostreae* had an individual effect on the expression of Ubiq (GLM, $F_1 = 5.513$, $P = 0.023$) and ACT (GLM, $F_1 = 6.634$, $P = 0.013$). The presence of *B. ostreae* was not found to have a significant effect on the expression of OeIAP (GLM, $F_1 = 0.217$, $P = 0.643$). Loch Ryan oysters had visibly higher expression of OeIAP than those from The Solent during the summer, but this was highly variable with a range of 9.15 in comparison to The Solent population with a range of only 3.76.

Effect of oyster origin

The interaction between oyster origin and season explained statistically significant amounts of variation in expression of OeIAP (GLM, $F_3 = 3.209$, $P = 0.030$), and Ubiq (GLM, $F_3 = 6.085$, $P = 0.001$), which suggests that the difference in expression of OeIAP and Ubiq between oysters from each origin depended on the season. Comparatively higher expression of Ubiq was seen in Solent oysters in Spring, and Loch Ryan oysters in the Summer. The interaction between populations throughout the seasons had less effect on the expression of OeC1q (GLM, $F_3 = 2.632$, $P = 0.059$). One Solent oyster showed very high expression of OeC1q in comparison to the other replicates and to the entire data set. This data point was not treated as an outlier, although it is likely to have influenced the outcome of the statistical analysis.

Oyster origin did not have an individual effect on OeFAS, which could indicate that OeFAS is involved in immune defence against *B. ostreae*. Although origin did not explain statistically significant amounts of variance, the expression of OeFAS does not appear to change much within the oysters from Loch Ryan across all four seasons. In comparison, expression of OeFAS was visibly increased in Solent oysters during spring and summer in comparison to that seen in the winter and autumn. Oyster origin did explain statistically significant amounts of variation in OeEcSOD expression (GLM, $F_1 = 5.333$, $P = 0.025$), and ACT (GLM, $F_1 = 4.009$, $P = 0.050$). Average expression of OeEcSOD was visibly higher in the Loch Ryan oysters than the Solent oysters during summer and autumn.

Effect of season

The expression of each gene was seasonal, and often depended on oyster origin or presence of *B. ostreae*. Expression of ACT was influenced by season (GLM, $F_3 = 6.992$, $P = 0.000$), and a post hoc pairwise comparison least square means test with Tukey's adjustment suggested that significantly lower expression of ACT was seen in winter in comparison to other seasons ($P < 0.05$).

Multivariate analysis

To visualise the entire data set, a principle component analysis (PCA) was conducted to look at the correlation between populations and seasons characterised by their expression of the six genes. The data were normalised to account for the different variance within each gene, and then fed into a PCR, visualised as a scatter plot (figure 2).

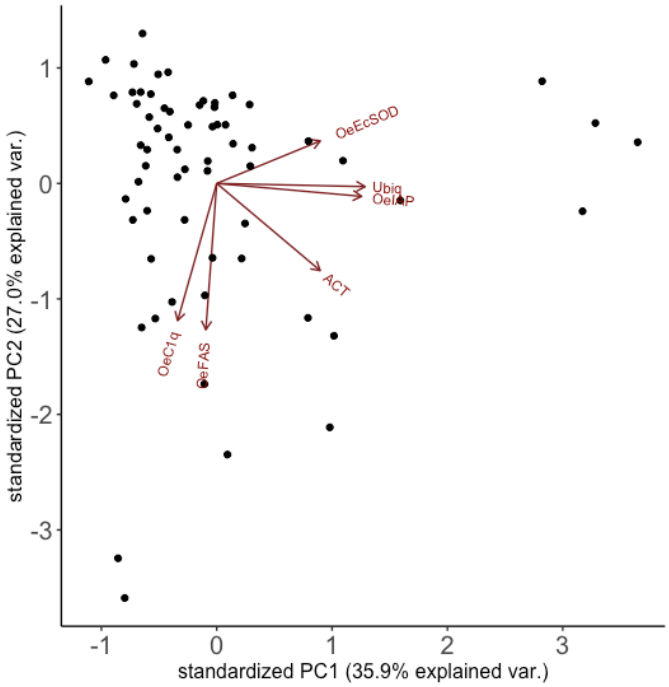
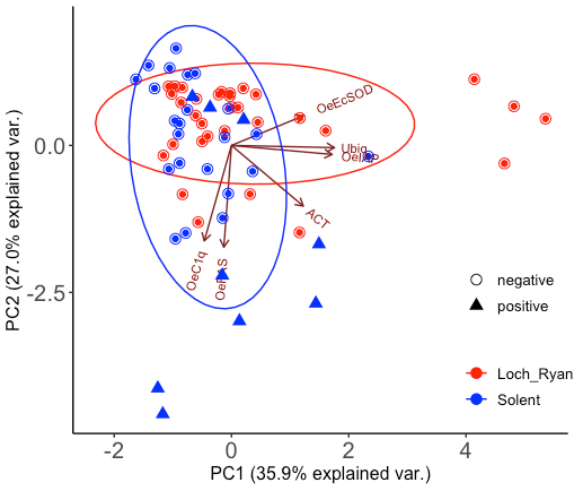


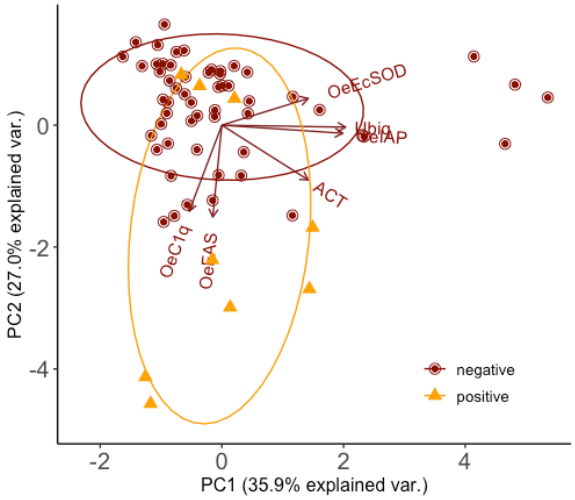
Figure 2. A PCA to observe the spread of (normalised) data, characterised by expression of six genes.

The most highly correlated genes were OeIAP and Ubiq (correlation = 0.71) and OeC1q and OeFAS (correlation = 0.43). The data were grouped by oyster origin (figure 3a), *Bonamia* presence (figure 3b), season (figure 3c), and by oyster batch (figure 3d).

(a)



(b)



(c)

(d)

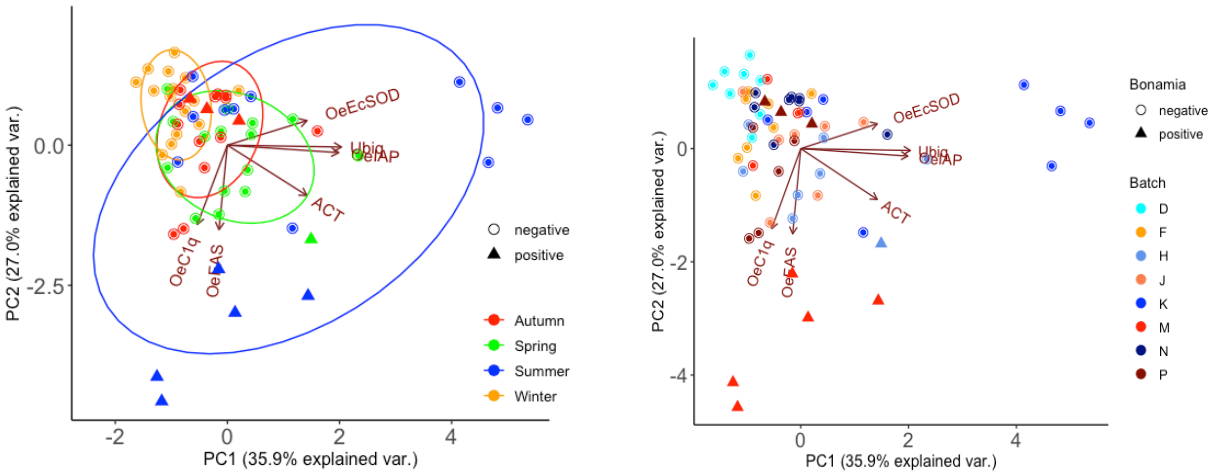


Figure 3. PCA representation of the correlation between populations (3a), *Bonamia ostreae* presence (3b), season (3c), and by oyster batch (3d) when characterised by gene expression (all normalised to have mean of 0 and sd of 1). *B. ostreae* positive individuals are indicated with a triangle. When divided into different oyster batches (3d), Solent oysters are represented in blues and Loch Ryan oysters in reds.

Interestingly, Loch Ryan oysters appeared to be more significantly characterised by expression of OeEcSOD, Ubiq and OeIAP than the Solent population, who appear to be more characterised by their expression of OeC1q and OeFAS. The highest variability in gene expression was seen during summer (fig 3d).

Discussion

Since its arrival to the south coast of England in 1982, *B. ostreae* has contributed to the disproportionate decline in the Solent *O. edulis* population and is still present in many surviving individuals. The ability of these oysters to tolerate and live with, or actively resist the *B. ostreae* infection has motivated further research into the *O. edulis* immune system and physiological pathogen defence mechanisms. *B. ostreae* has not yet been reported in the Loch Ryan wild oyster fishery. This has provided an opportunity to compare some physiological mechanisms of *Bonamia*-exposed and *Bonamia*-naïve *O. edulis* in two working fisheries through analysis of their gene expression and metabolome. Morga et al (2017) identified a number of genes thought to be associated with *Bonamia*-resistance, but compared these using only one reference gene, neglecting approved MIQE compliance regulations. In this study, the expression of Ubiq, ACT, OeIAP, OeEc-SOD, OeC1q, and OeFAS was compared between oysters from Loch Ryan and the Solent during all four seasons in 2018, using four reference genes: GAPDH, Ef1-α, L5, and Cathep. Gill tissue from each oyster was tested for the presence of *B. ostreae* in order to compare gene expression between infected and non-infected oysters. Hepatopancreatic tissue, taken from the same oysters, was used to assemble a metabolome profile for each individual.

Increased expression of an inhibitor of apoptosis (OeIAP), Fas ligand (OeFAS), and extracellular superoxide dismutase (OeEcSOD) have previously been found in *Bonamia*-resistant flat oysters (Morga *et al.*, 2012). C1q

proteins are involved in recognition of pathogens by the invertebrate innate immune system (Zhang *et al.*, 2008; Gerdol, Venier and Pallavicini, 2015), and OeC1q has previously demonstrated different expression in *O. edulis* due to bonamiosis (Martín-Gómez *et al.*, 2014; De La Ballina, Villalba and Cao, 2018). Although β-actin (ACT) and polyubiquitin (Ubiq) are occasionally identified as stable reference genes (Morga *et al.*, 2010; Wang *et al.*, 2013), enough variation was seen between our samples (by qbase) to require their comparison between treatments.

The effect of season explained statistically significant amounts of variance in gene expression, which only reinforces the importance of long-term studies within the discussion of *Bonamia*-resistance. As expected, *B. ostreae* was only found in the Solent population, yet no individuals were found to be infected with the parasite during the winter. This coincides with previous reports finding lower pathogen concentration at this time (Zabaleta and Barber, 1996; Lynch *et al.*, 2007; Madsen, Kamp and Mellergaard, 2013). It has been suggested that oysters are more susceptible to infection during the warmer months of the year due to during or post-spawning or post-settlement stress (late spring-early autumn) (Walne, 1961; Hine, 1991; Engelsma *et al.*, 2010), which is well documented in Crassostrea spp. (Samain and McCombie, 2008; Huvet *et al.*, 2010; Wendling and Wegner, 2013). This coincides with previous reports that have found a correlation in temperature and *Bonamia* prevalence; lower temperature results in lower prevalence of *Bonamia* (Hine, 1991; Carnegie *et al.*, 2008). Temperature always plays an important role in the prevalence and spread of pathogens (Snieszko, 1974), and the effect is evident here in the form of seasonal fluctuations.

Morga *et al.*, (2012) observed higher expression of OeIAP, OeFAS and OeEcSOD in *Bonamia*-resistant oysters in comparison to wild oysters, but down-regulation of OeEcSOD in previously *Bonamia*-naïve oysters 1 h after injection with pure *B. ostreae*. Although both oyster populations in this study were from wild fisheries, the comparison between oysters from a *Bonamia*-naïve area with those from a *Bonamia*-exposed area gave us the chance to closely observe the effect of the parasite on *O. edulis* immunity. Our results certainly show a higher expression of OeFAS in the Solent population throughout spring, summer and autumn when *B. ostreae* is present amongst the population. This was obvious in comparison to the expression of OeFAS amongst that of the Loch Ryan population that remained relatively stable throughout the year. During the summer, the Solent oysters with the highest OeFAS and OeIAP expression were also the oysters with a *B. ostreae* infection, which could represent a correlation between these genes and an immune response. However, this became reversed during autumn, where the Solent oysters with a *B. ostreae* infection were expressing lower levels of OeFAS and OeIAP than the non-infected oysters. If temperature has an influence on prevalence of *B. ostreae*, then perhaps this suggests that OeFAS and OeIAP are significant genes for immune defence specific to *B. ostreae*, as expression is elevated during the months when *B. ostreae* would be most effective and prevalent. The Loch Ryan population had visibly higher OeIAP and OeEc-SOD expression during the summer, but also a large range suggesting high variability amongst the population. Inhibitors of apoptosis (IAP's), found in both invertebrates and vertebrates, are responsible for regulating apoptosis, a common immune response to pathogen cell invasion. It is therefore not surprising that OeIAP was upregulated

during summer months when disease and a variety of pathogens are more prevalent. Morga *et al.*, (2012) found OeIAP to be elevated in a *Bonamia*-resistant population, yet the highest levels of expression seen in this study were from the *Bonamia*-naïve Loch Ryan population and not the *Bonamia*-exposed Solent population. This could indicate either that this gene has a role not specifically or directly related to defence against *B. ostreae,* or that the Loch Ryan oysters naturally have some form of *Bonamia*-resistance.

The interactive effect of season and presence of *B. ostreae* explained statistically significant amounts of variance in the expression of OeEc-SOD (associated with oxidative detoxification), but completely mirrored the results seen by Morga *et al.*, (2012) with higher expression seen by Loch Ryan oysters that had not been exposed to the parasite. A significant difference in expression of OeC1q was seen in oysters from *Bonamia*-naïve (Loch Ryan) and *Bonamia*-exposed (Solent) populations and the concentration depended on season (and presence) of *B. ostreae*. OeC1q is from the family of C1 proteins responsible for immunity against pathogens in vertebrates (Kishore and Reid, 2000) and invertebrates (Zhang *et al.*, 2008), and are considered reliable markers for resistance in other organisms such as clams (*Mercenaria mercenaria*) to diseases such as Quahog Parasite Unknown (QPX) (Perrigault, Tanguy and Allam, 2009; Ronza *et al.*, 2018). One *Bonamia*-infected oyster from the Solent population had a disproportionally high concentration of OeC1q in the summer, which (if we are to consider this gene as a resistant marker) could be an example of the level needed to contribute to *Bonamia*-resistance of this individual to the pathogen.

The polypeptide Ubiquitin has roles in both innate and specific immunity of organisms from plants to the most complex animal (Goldstein *et al.*, 1975). This conservative protein been recognised in multiple immunity associated processes such as initiating the synthesis and degradation of other proteins involved with apoptosis of viral cells (Yan and Chen, 2012), differentiating between lymphocytes, and modulating signalling pathways or transcription factors (Liu, 2004). Ubiquitin is recognised as an important component in the coordination of signals responsible for immune mechanisms such apoptosis and inflammation (Skaug, Jiang and Chen, 2009), and therefore it is not surprising that a higher concentration of Ubiq was seen in *Bonamia*-exposed (Solent) oysters during spring and summer when oysters were more vulnerable to *B. ostreae* infection. However, because Ubiq has already been established as a gene associated with innate immunity in invertebrates, it would be necessary to compare the expression of Ubiq in oysters infected with a different disease to these *B. ostreae* infected oysters to determine an immunity role specific to *B. ostreae*.

Phagocytosis is essential to innate and specific immune response and is a process dependent on the family of actin proteins (May and Machesky, 2001; Li and Xiang, 2013). Beta-actin (ß-actin or ACT) is a cytoskeletal protein involved in cell structure and motility, and embryonic development (Bunnell *et al.*, 2011). Although this conservative protein is often considered a stable reference gene, ACT has more recently been recognised as an essential component in innate antiviral immune response by regulating specific proteins (Xie *et al.*, 2019), and contributing to important protein complexes involved with invertebrate immune defence such as that found in shrimp against white spot syndrome virus (WSSV) (Li and Xiang, 2013). Xie *et al.*, (2019)

found that the absence of mitochondria-targeted ACT was detrimental to the innate antiviral immune signalling that initiates an immune response, which suggests that ACT plays a much larger role in invertebrate immunity than previously thought. In this study, expression of ACT was significantly different between populations, seasons and oysters with and without a *B. ostreae* infection. This variability demonstrates the unsuitability of the repeated use of ACT as a housekeeping reference gene, and suggests it is an influential component of *O. edulis* immunity encouraging further analysis into its specific role against a *B. ostreae* infection.

Conclusion

Investigation into six genes associated with *O. edulis* immunity showed a significant difference in the expression of ACT, Ubiq, OeC1q and OeFAS between *Bonamia*-exposed oysters and *Bonamia*-naïve oysters. However, significant seasonal variation in the expression of all six genes tested demonstrates a need for long term and seasonal studies in the battle to identify *Bonamia*-resistant markers in *O. edulis*. We urge supplementary research to rigorously follow MIQE compliant qPCR methods in order to further this research with robust results that will best benefit restoration practice.

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