The immunobiology of *Crepidula fornicata* and its potential to act as a harbourer of infectious disease

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This dissertation is submitted for the degree of

Doctor of Philosophy in Biological Sciences to Swansea University

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Declarations

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Ethics Statement

Approval Number: SU-Ethics-Student-111217/446

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Prior to conducting this research, Swansea University's Research Ethics and Governance Framework was read.

This project makes use and generates new data. This study did **NOT** pose a potential risk to the environment, such as the escape of invasive species, genetically modified organisms (GMO), work involving human or animal pathogens, environmental contaminants, radioactive material, or active outdoor vegetation fires. This study also did **NOT** involve humans as the focus of research.

This study was not interventional; it did **NOT** involve the capture, handling, and / or confinement of living vertebrates or cephalopods.

No conflicts of interest are to be declared.

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Emma Quinn

Abstract

Invasive alien marine species have garnered a negative reputation outside of their native ranges, often due to them translocating disease from their native areas to their new environment. Much of the currently available resources on the disease status of marine species is focused on species of commercial importance such as the Blue mussel *Mytilus edulis*, Pacific oyster *Crassostrea gigas* or farmed abalone species e.g., *Haliotis* spp.

The slipper limpet *Crepidula fornicata* is an invasive alien species, originating from the east coast of the United States. *Crepidula fornicata* is now found throughout the coastal waters of southern England and Wales, U.K. *Crepidula fornicata* is often thought of as a pest when found in commercial shellfish areas. However, despite this negative reputation, little is known about its disease status or immunobiology. Therefore, the aim of this thesis was to study the immunobiology of *C. fornicata and* to understand its potential as a harbourer of infectious disease.

To address the lack of information available with regards to the immunobiology of *C. fornicata in* **Chapter 2** haemolymph (blood) was isolated and examined for the presence of the immune-enzyme phenoloxidase. The presence of laccase and catecholoxidase activities were confirmed. Importantly, it was shown that products derived from laccase and catecholoxidase activities reduced the numbers of colony- forming units of bacteria *in vitro*. Tissue was also examined histologically, and the presence of eumelanin-like pigments and lipofuscin was visualised in a number of regions e.g., digestive gland, connective tissues, and gills.

A year-long multi-resource disease survey was carried out at two sites in South Wales, Swansea Bay, a native oyster *Ostrea edulis* restoration site, and Milford Haven, an area of commercial shellfish activity. In **Chapters 3 & 4** a combined PCR and histological approach was taken to screen *C. fornicata for* the presence of diseases potentially harmful to shellfish and/or humans e.g., Vibrios, haplosporidians, microsporidians, and paramyxids. **Chapter 3** found that a large proportion of individuals were PCR positive for Vibrio-like bacteria. However, it was found through a histological screen that few disease signatures could be observed, suggesting that *C. fornicata are* not particularly sensitive to bacteriosis at the sites surveyed. **Chapter 4** found no clinical signs of *C. fornicata being* infected with haplosporidians, microsporidians or paramyxids. Histology revealed the presence of trematodes, turbellarians, and an apicomplexan-like parasite. The data suggested that *C. fornicata are* not susceptible to major parasitic infections outside of the native range.

A Species Distribution Model (SDM) was constructed in **Chapter 5** to aim to understand the potential future distribution of *C. fornicata under* predicted climate change. Areas further north in North America, Europe, and Northeast Asia were identified as areas most at risk for future introductions of *C. fornicata.*

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4-HA- 4-hydroxyanisole;
4-HR - 4-hexylresorcinol;
ABTS - 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AUC – Area Under the Curve
BLASTn - Basic Local Alignment Search Tool
BLRM – Binomial Logistic Regression Model CFU - Colony forming units
CLRs – C-Type lectin receptors
CO - Catecholoxidase;
CTAB - Cetrimonium bromide;
DHPPA - 3,5-Dihydroxyphenylpropionoicacid;
EASIN – European Alien Species Information Network
EDTA - Ethylenediaminetetraaceticacid;
GBIF – Global Biodiversity Information Facility
HC – Haemocyte Counts
IPCC – Intergovernmental Panel on Climate Change
L-DOPA - 3,4-Dihydroxyphenylalanine;
LPS – Lipopolysaccharide
Maxent – Maximum entropy
MH – Milford Haven
MI – Maximum Identity
OBIS – Ocean Biogeographic Information System
PAMPs – Pattern Associated Molecular Patterns
PCR – Polymerase Chain Reaction
PO - Phenoloxidase;
PPD - para-Phenylenediamine;
PRRs - Pattern recognition receptors
PTU - Phenylthiourea;
QC – Query Coverage
QX disease – Queensland Unknown Disease
RCP – Representative Concentration Pathway
RIG – RIG like receptors
SB – Swansea Bay
SDM - Species Distribution Model
SRA – Short Read Archives
SST – Sea Surface Temperature
Syringaldazine - 4-Hydroxy-3,5-dimethoxybenzaldehydeazine;
TBE – Tris/Borate/EDTA
TCBS – Thiosulfate Citrate Bile salts Agar
TCR – Toll-like receptors
TSA – Tryptone Soya Agar
TSS – True Skill Statistics
TY - Tyrosinase

Chapter 1 General Introduction

1.1 Background

Crepidula fornicata (Linnaeus, 1758) is an invasive alien marine gastropod in the Calyptraeidae family. It is native to the east coast of the United States but has successfully invaded a substantial proportion of the European coastline over the last century. It was first accidentally introduced into UK coastal waters with shipments of the American oyster (*Crassostrea virginica;* Yonge & Thompson, 1976) and is now commonly found along the coasts of France, the Netherlands, Germany, Denmark, and Norway (Blanchard, 1997). Specimens have also been reported in Irish waters (McNeill *et al*., 2010). *Crepidula fornicata,* commonly referred to as the slipper limpet, is a suspension feeder, filtering phytoplankton and particulate organic matter. Along European coasts, *C. fornicata* is an oyster pest. It is commonly found on coastal infralittoral grounds, however stranded populations of *C. fornicata* can be seen on the lower part of the neritic zone of exposed shores. The maximum depth at which it is commonly found is 30 m (Barnes *et al*., 1973). Populations tend to be at their largest in wave protected areas such as estuaries, bays or on the inland side of exposed islands. Due to *C. fornicata* being ubiquitous, eurythermal and euryhaline, they can be found in a range of environments, i.e., rocky, gravel or sandy bottoms, as well as in muddy areas, where densities tend to be at their highest (Blanchard and Hamon, 1994).

Two specific characteristics of *C. fornicata* have resulted in numerous studies. The first characteristic relates to its behaviour. Individuals settle on top of each other forming stacks. The second characteristic is that *C. fornicata* are protandrous (sequential) hermaphrodites. These characteristics along with several other life history traits have been shown to have helped contribute to its successful introduction and spread along European coastal waters. The life history traits that have aided in the introduction of *C. fornicata* to European coastal waters, include: the strong potential for natural dispersal during the pelagic life stage, and having the capability to extend pelagic life further when environmental conditions are unsuitable e.g decreased temperature and salinity levels (Bashevkin and Pechenik, 2015; Pechenik, 1984; Viard *et al*., 2006). Other factors that have aided in their spread include the strong potential for dispersal due to anthropogenic activities, such as the movement of ballast water, ship hull fouling and the translocation of shellfish for aquaculture (Crouch, 1893; McMillan, 1939; Thieltges,

2005a). Due to the long reproductive season, females can spawn multiple times per year, further increasing their potential to spread along coastlines and increase in numbers (Richard *et al*., 2006).

Figure 1.1 *Crepidula fornicata* shells found in extremely high numbers along Swansea Bay, Wales

The arrival of *C. fornicata* to the coasts of South Wales (Figure 1.1) has had a major negative impact on local waterways, fish hatcheries, and shellfish beds (Bohn, 2012). Environmental costs resulting in disruptions to the ecosystem structure, function and biodiversity have extended to social impacts including risks to human health and safety, recreational opportunities, and cultural heritage. Although many studies have taken place on *C. fornicata* concerning its ecology, little is known about its disease harbouring potential or immunobiology.

1.2 Distribution of *Crepidula fornicata*

The introduction of invasive alien species (IAS) is a major driving force behind ecological and evolutionary changes and are a challenge for the conservation of biodiversity and economic impacts in their new environment (Grosholz, 2002). IAS can

cause new species interactions but can also alter existing ones. Invasive alien species have the potential to affect all levels of ecological organisation: individuals, populations, communities and ecosystems (Jeschke *et al*., 2014). Colautti and MacIsaac (2004) suggest that the term *invasive* be used for any IAS that have successfully passed the main phases of the invasion process and became widespread in the non- native range. The Invasive Non-Native Species Framework Strategy for Great Britain defined IAS as species "whose introduction and/or spread threaten biological diversity or have other unforeseen impacts" (DEFRA, 2015)

The genus *Crepidula* contains around 60 species (Henry *et al*., 2010), with the majority being native to the American coasts (Hoagland, 1977). Only *Crepidula unguiformis* (Lamarck, 1822) and *C. gibbosa* (Defrance, 1818) are native to Europe, being present in the Mediterranean and Adriatic seas (Hoagland, 1977). All other species found along European coasts are introduced. A single specimen of *Crepidula plana* (Say, 1882) was found in Ireland, in 1865, but never became introduced in Europe (Minchin *et al*., 1995). *Crepidula calyptraeiformis* (Deshayes, 1830) became introduced into Alacant Harbour, Spain from South America (Zibrowius, 1991). *Crepidula fornicata* has been the species with the most success outside of its native range. It is native to the North American Atlantic coast, with a wide distribution ranging from the Gulf of St. Lawrence, Canada to the Gulf of Mexico and on the Caribbean islands (Fretter and Graham, 1981). *Crepidula fornicata* was first recorded outside of its native range in 1872, when a specimen was found in Liverpool Bay, England (McMillan, 1939). The most probable mode of introduction was that it was attached to either the American clam (*Venus mercenaria* Linnaeus, 1758, or the American oyster *C. virginica*), both species that were being imported into this area at the time. Reports of *C. fornicata* in Beaumaris in North Wales around 1886 were also made, again, associated with *C. virginica*. However, it appears that these populations were not able to persist (Barnes *et al*., 1973). Records of *C. fornicata* were not kept on the British west coast until the 1950s (Cole and Baird, 1953). Due to the British oyster trade collapsing because of a fall in stocks of the native oyster (*Ostrea edulis;* Linneaus, 1758), there were large imports of *C. virginica* to Britain (Blanchard, 1997). This movement of *C. virginica* allowed the repeated introduction of adult *C. fornicata* to British waters, especially to the east and south coasts of England. Several live and dead *C. fornicata* shells were found in the Colne, Crouch and Roach

rivers in Lincolnshire and Essex between 1887-1893 (Crouch, 1893; Adam and Leloup, 1934). In a matter of years, *C. fornicata* became abundant in these areas. *Crepidula fornicata* was first recorded in Ireland with imports of the American oyster arriving in Ballinakill Bay on the west coast of Ireland, but were removed before the oysters were laid on the shore (cited in Sykes 1902; McNeill *et al*. 2010). There is a possibility that living specimens had been introduced to Carlingford Lough by the same means in the early 1920s, as six worn shells appeared in the National Museum of Ireland, Dublin labelled NMI 130.1925. However, no living specimens were recorded in Carlingford Lough until the European open trade agreement, in operation from January 1993, a period of time when imports of Pacific half-grown osyters, with a number of small male *Crepidula fornicata* attached to oyster shells, were imported from France (Minchin *et al*., 1995). Males were also found on Pacific osyter imports to Dungarvan and Carlingford bays. The oysters were held in meshed bags on trestles. The oysters were re-examined a number of months later, and no specimens were found, there were likely to have been crushed by the turning of osyters with the bag (McNeill *et al*., 2010). There is a possibility that populations persisted in Kilmakilloge Harbour and Clew Bay, until an extremely cold winter in 1962/1963 resulted in high mortalities. *Crepidula fornicata* did not become established in Ireland until recently. *Crepidula fornicata* was confirmed in Belfast Lough in 2009, when over 20 specimens were found intertidally (McNeill *et al*., 2010).

Other than the records of *C. fornicata* on the west coast of Britain from the 1880s, it had not been recorded again on the west coast of Britain until 1953. Six specimens were reported in Pennar Gut in the Milford Haven Waterway, Wales (Cole and Baird, 1953). Both stacks and egg-brooding females were found, implying that they had the ability to reproduce. The most likely mode of introduction in this area was through *C. fornicata* attaching itself to the underneath of naval and merchant ships that were brought to the Milford Haven Waterway for repair and breaking up after staying in *C. fornicata* populated areas on the east and south coast of England (Cole and Baird, 1953). Both intertidal and subtidal populations of *C. fornicata* increased in numbers rapidly.

Crepidula fornicata is now well established in both the South and Southwest of Wales (Figure 1.2). It has reached particularly high abundances in the Milford Haven

Waterway, the original introduction site. It is also commonly found in Swansea Bay (Bohn, 2012), The northward expansion of *C. fornicata* range in the U.K has been limited. During the 2008 scallop survey of the Skomer Marine Conservation Zone, just north of the Milford Haven Waterway, two specimens were found attached to live *Pecten maximus* (Linnaeus, 1758) at one site (Lock *et al*., 2012). Subsequently, during the 2016 scallop survey at Skomer, 68 specimens were found attached to scallop shells, as stacks or individuals, at two sites, along the north Marloes Peninsula and at a site on the north side of Skomer Island (Newman *et al*., 2016). This suggests that *C. fornicata* may have a limited breeding population at Skomer.

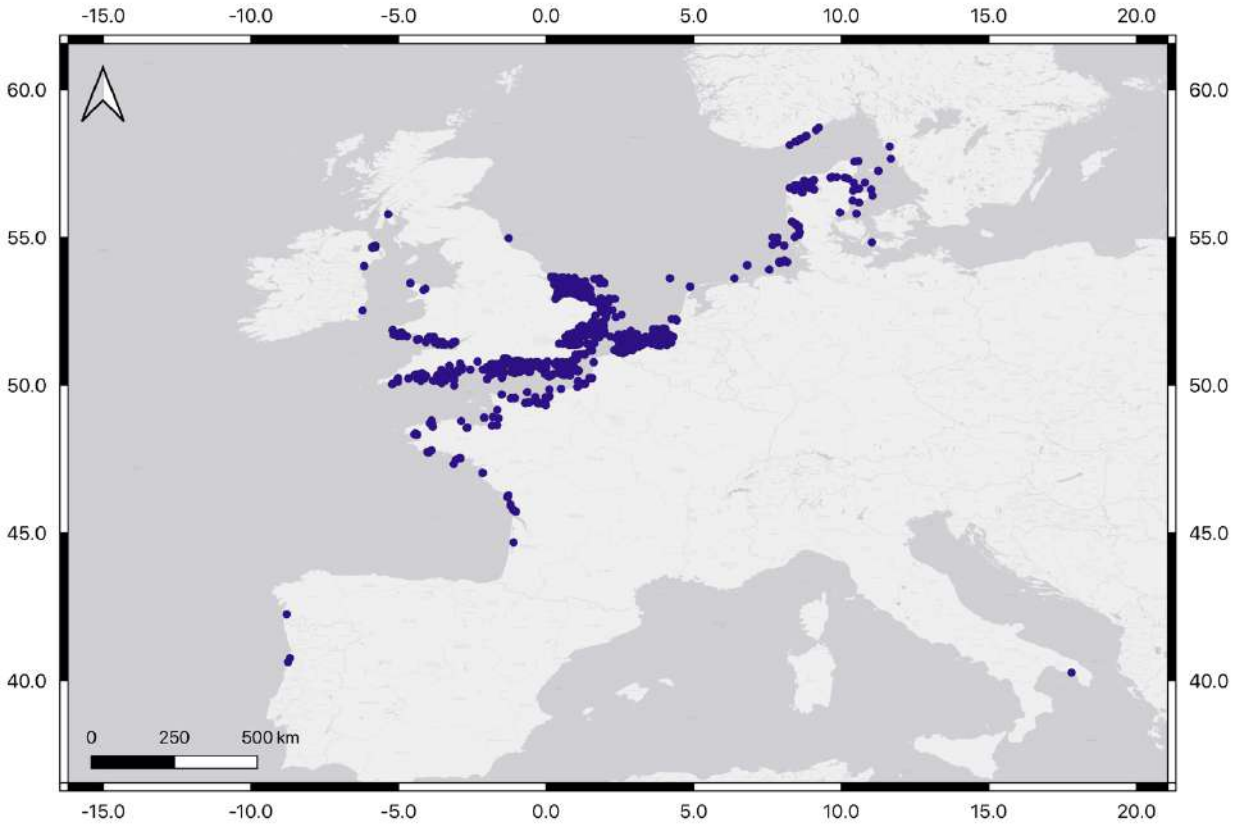

Figure 1.2 Current records of *Crepidula fornicata* along the European Coastline (Data: Ocean System; 3.10 Coruña)

1.3 *Crepidula fornicata* biology

1.3.1 Genetics

A study carried out by Riquet *et al*. (2013) studied 683 individuals from seven native ranges in the U.S.A. and 15 introduced populations including those in England, and Wales. The study confirmed high genetic diversity in both native and introduced

populations with a lack of genetic structure between the two ranges. The study found that three populations from the UK (Lawrenny, Portsmouth and Canvey Island), one French population (Port-en-Bessin) and an American introduced population (Mud Bay, Washington), showed a similar genetic composition, contrasting with other European populations. The reported mode of introduction of *C. fornicata* to the U.K through the movement of American oysters (Blanchard, 1997) is supported by the genetic similarities between UK populations, and native populations in the U.S.A. The genetic similarity between the other European populations from Sweden (Tjarno), Denmark (Limfjord), Germany (Sylt Island), the Netherlands (Yerseke), and France (Gravelines, Bay of Morlaix, Bourgneuf, Fouras, Arachon, and Sete) in the study, is likely to be as the result of the repeated release of *Crassostrea gigas* (Thunberg, 1793) spat in the 1970s (Grizel and Héral, 1991). *Crassostrea gigas* previously introduced in the North Pacific, an area where *C. fornicata* had previously been imported, were introduced along the Mediterranean and Atlantic coasts of France (Blanchard, 1997). *Crassostrea gigas* continued to spread to northern Europe potentially with *Crepidula fornicata* (Smaal *et al*., 2009).

1.3.2 Life cycle and reproduction

Crepidula fornicata are protandrous hermaphrodites (Orton, 1952). Sequential hermaphroditism is a reproductive strategy by which an organism changes sex during their lifetime (Hoch and Cahill, 2012), in protandrous hermaphrodites, organisms are predicted to change sex from male to female when the relative reproductive fitness of females overtakes that of males (Hoch and Cahill, 2012). Protandrous hermaphroditic males are younger and smaller, and when they reach the size at which females have a higher relative fitness, they change sex (Ghiselin, 1969). The reproductive value of an individual is dependent on a number of factors, including both current and future reproductive outputs and the probability of surviving in order to successfully reproduce in the future (Broquet *et al*., 2015). Therefore, there are various mechanisms that can cause males and females to show different reproductive value trajectories due to size/age.

Females tend to produce larger gametes; this production and storage of these gametes necessitates space within the body. Also, the production of larger female gametes has a higher energy cost that for males. Therefore, female fecundity in many species

increases with body size. If this effect of size results in larger individuals having a higher reproductive value as females, then protandry is selected first i.e. male (Broquet *et al*., 2015). Sex-linked differences in terms of growth and mortality rate can cause the effect of size on reproductive value being higher in one sex than the other, therefore selection for sex change becomes introduced. Sex-change theory shows that in can be advantageous to start life as the sex that has the fastest growth rate and/or lowest mortality rate (Charnov, 1982; Iwasa, 1991). Social systems can also result in conditions where a differential effect of size on male versus female reproductive effect can be created (Munday *et al*., 2006).

Protandrous sex-change in the genus *Crepidula,* is a well-documented display of sequential protandrous hermaphrodism (Orton, 1909; Coe, 1936, 1938; Chipperfield, 1951). Slipper limpets form long-lived stacks of anywhere from 2-20 individuals, with younger/smaller individuals (immature/male) pilling up on older/larger individuals (female) (Coe, 1936). These stacks are permanent mating associations of genetically unrelated individuals (Broquet *et al*., 2015). All slipper limpets begin life as male before changing sex. A study carried out by Cahill *et al*. (2015) investigated the social conditions under which this sex change occurs in *Crepidula fornicata* by altering physical and chemical contact with conspecifics. This was achieved by allowing males to be either in physical and chemical contact with females or in chemical contact with, but physically isolated from, females. The study found that for males that were in physical contact with females, were less likely to change sex than the isolated controls, whilst males in chemical (but not physical) contact with females changed sex at the same rate as the isolated controls. These results demonstrate that the factor controlling sex change in *C. fornicata* is as a result of contact-borne inhibitor associated with female conspecifics (Cahill *et al*., 2015).

Parentage studies in *Crepidula fornicata* have shown that the mating system is polygamous and that the reproductive success of the male can be highly variable (Dupont *et al*., 2006; Proestou *et al*., 2008; Le Cam *et al*., 2009;). This variability appears to have links with the position of an individual within a stack, the stacking process means that larger, older males are closer to the females, and tend to have higher reproductive success (Dupont *et al*., 2006; Proestou *et al*., 2008).

Another important mating system used by *Crepidula fornicata* is that of sperm storage. Female *C. fornicata* can mate multiple time and produce numerous broods over a reproductive season using sperm from multiple males that has been stored, potentially from at least 1 year (Broquet *et al*., 2015). In sequential hermaphrodites that utilise internal fertilization, sperm storage has the potential to the reduce the cost of changing sex, and could affect the fitness of both males and females (Broquet *et al*., 2015).

Crepidula fornicata begins life as free-swimming larva for 2-7 weeks, and can live for up to 10 years in a sessile adult form (Riquet *et al*., 2017). Slipper limpets have a high fecundity, with females capable of releasing 5000-25000 swimming veliger larvae, two to four times per year on average (Richard *et al*., 2006). Larvae metamorphose and settle at a length of 800-1000 µm on a hard substrate, often on *C. fornicata* adult stacks (Bohn *et al*., 2015). *Crepidula fornicata* demonstrate a stacking behaviour, whereby males stack on top of females, with a sex change occurring from male to female according to individual age and stack sex ratio (Coe, 1938; Le Cam and Viard, 2011). Reproduction can span from two to eight months (Henry *et al*., 2010). *Crepidula fornicata* reproduction takes place through internal fertilization, mostly between males and females of the same stack, with each stack forming a local breeding group (Proestou *et al*., 2008; Broquet *et al*., 2015;). *Crepidula fornicata* have demonstrated multiple paternity, and sperm storage (Le Cam *et al*., 2009; Beninger *et al*., 2010). Developing embryos after fertilization are encapsulated and brooded (Hoagland, 1977), until swimming veliger larvae are released at a size of ~ 400 µm (Pechenik *et al*., 2002).

1.3.3 Physiology and phenology

Feeding

Crepidula fornicata is a suspension-feeding species, consuming phytoplankton during both the pelagic larval phase and as an adult (Pechenik and Strathmann, 2017). The feeding mechanism, however, are different between the two stages. Like many marine gastropods, *C. fornicata* hatches as a veliger and swims and feeds for a number of weeks before metamorphosing. Veliger slipper limpet larvae develop part of the adult feeding apparatus, which includes ctenidial filaments, neck lobe, and radula, before metamorphosis, however ctendial feeding does not being until after the loss of the larval feeding apparatus (velum) at metamorphosis (Pechenik and Strathmann, 2017).

Veligers capture food particles between two bands of cilia at the edge of each velar lobe that beat towards each other. Captured particles are transported in the direction of the mouth along a ciliated food groove found between the opposed bands. (Pechenik and Strathmann, 2017).

Shumway *et al*. (2014) captured the collection of food particles by adult *Crepidula fornicata* and documented the feeding cycle through the combined usage of a specially designed video system paired with video endoscopy and microscopic observations. Video endoscopy showed that slipper limpets capture food particles on the frontal surface of the filaments and are moved distally via the frontal cilia of the gill. After capture, particles become incorporated into fine mucous strings on the frontal surface and carried both obliquely and distally across the filaments. At the distal edge of the gill, the mucous strings enter the neck canal, form a food cord, and pulled anteriorly, resulting in the oblique movement of material on the gill.

Blanchard *et al*. (2008) studied the degree of which *Crepidula fornicata* and *Crassostrea gigas* may compete for food. The study found that slipper limpet larvae ingested phytoplankton over a wider range of cell size and ate at higher rates (up to twice the rate) on each tested diet, when compared to that of *C. gigas*. The study highlighted that intensive grazing by slipper limpet larvae could potentially deplete phytoplankton concentrations resulting in competition with oyster larvae.

1.3.4 Ecological impacts of *Crepidula fornicata*

Trophic competition

Crepidula fornicata is a suspension feeder filtering around 0.76 ± litre $h^{-1}g^{-1}$ (Barillé *et al*., 2006). If populations reach dense levels, this can result in the diversion of large quantities of phytoplankton and organic matter, potentially impacting the available concentration and resulting in trophic competition with other suspension feeders (Orton, 1926; Blanchard, 1997) . However, it appears that in most areas, no mortality is observed in other species due to the lack of food (de Montaudouin *et al*., 1999).

Spatial competition

Crepidula fornicata stacks if found in high densities, can prevent other larvae and juveniles from settling and spatial competition can occur. Le Pape *et al*. (2004) studied

the effects of *Crepidula fornicata* on habitat suitability for juvenile common sole *Solea solea* in the Bay of Biscay and found that though there was no apparent effect on the extent of the sole nursery grounds, the density of young-of-the-year sole was significantly lower there *C. fornicata* was established. Kostecki *et al*. (2011) saw similar effects in Mont-Saint-Michel Bay, France, whereby the rapid expansion of *Crepidula fornicata* in the bay has resulted in the availability of suitable surface for flatfishes (common sole *Solea solea;* plaice *Pleuronectes platessa;* brill *Scophthalmus rhombus* and flounder *Platichthys flesus*) become restricted to only the eastern part, where previously, the entire bay was a suitable habitat.

Impacts on habitat

Due to the filtrating activity of *Crepidula fornicata*, it was found that this resulted in the accumulation of pseudofaeces and fine sediment. The stacking nature of *C. fornicata* results in stacks protruding into the water column. Potentially resulting in changes in sediments and near-bottom currents (Ehrhold *et al*., 1998). The production of mucoidal pseudofaeces can result in the benthic substrate changing from predominantly sandy to muddy with a high organic content that can quickly become anoxic and unsuitable for other species (Streftaris and Zenetos, 2006). Thieltges (2005b) found during field experiments designed to experimentally test the effects of *Crepidula fornicata* on survival and growth on the blue mussel *Mytilus edulis*, that epigrowth by *C. fornicata* resulted in a 4- to 8- fold reduction in mussel survival. Shell growth in surviving mussels with attached slipper limpets was 3 to 5 times lower when compared to unfolded mussels. The author suggests that interference competition in the form of changes in small-scale hydrodynamics by *C. fornicata* stacks as a causative agent.

1.3.5 Economical impacts of *Crepidula fornicata*

Dense populations of *Crepidula fornicata* can disturb some sea-bed oyster culture activities and some fisheries to such a large level that in some bays (e.g. Sheldt estuaries in Zeeland, Thames estuary and Fal river in the U.K, the Norman gulf or the Atlantic Marennes pond in France, that the clearing and removal of slipper limpets is required (Fitzgerald, 2007; Kostecki *et al*., 2011). If populations of *C. fornicata* reach high densities, oyster grounds need to be regularly cleared before sowing need seed (O'Rourke and O'Flynn, 2014). When *C. fornicata* becomes attached to the shells of commercial molluscs these require removal before sale (Blanchard, 1997).

1.4 Diseases of molluscs

Molluscs are the second-largest invertebrate phylum only after the Arthropoda, composed of ~ 85000 extant species (Chapman, 2011), with World Register of Marine Species (WoRMS) cataloguing just over 62000 marine molluscs (WoRMS Editoral Board, 2021). Molluscs can act as important ecosystem engineers, adding structure to aquatic bottom environments, providing habitat, protection, and food to a wide range of other groups (Fortunato, 2015). Molluscs importantly support significant fishery and aquaculture industries globally (Wijsman *et al*., 2018).

Aquaculture has a major role in global food security, producing 80.1 million tonnes of food in 2016 (Potts *et al*., 2021). Growth of the aquaculture sector is ongoing and is increasing as an important source of seafood as capture fisheries has remained stable since 1990 (FAO 2019). Molluscs e.g. bivalves, gastropods and cephalopods, account for 21.4% of all aquaculture globally (Potts *et al*., 2021). Bivalves e.g. oysters, clams, mussels and scallops account for the majority of molluscan aquaculture, gastropods such as abalone also make an important contribution (Venugopal and Gopakumar, 2017). Currently, cephalopod aquaculture has little commercial significance, potentially due to the lack of culture expertise, unknown optimal nutritional requirements and difficulties associated with animal reproduction in captivity (Vidal *et al*., 2014; O'Brien *et al*., 2018).

The European Union (EU) is a region which has a large market for aquaculture products, and is one of the world's largest importers of seafood (Fox *et al*., 2020). However, over the last 20 years despite the demand, aquaculture production within EU countries has declined, e.g. mussel production, volumes peaked in the late 1990s and had dropped by 20% by 2016 (Avdelas *et al*., 2021). The spread of disease has been one of the factors attributed to the decline in production, and has resulted in social and economic disruptions (Oidtmann *et al*., 2011). For the majority of molluscan aquaculture the grow-out phase occurs in the open ocean, and this can expose animals to a wide range of viruses, bacteria, fungi, protozoans, and metazoans (Bower *et al*., 1994; Lafferty *et al*., 2015; Arzul *et al*., 2017). Farmed species can often receive diseases from wild species, who in turn, can export infectious disease to wild species (Lafferty *et al*., 2015). Biotic and abiotic factors of environmental change can disturb host- parasite

interactions and can cause an increase or decrease in disease risk. Anthropogenic stressors such as climate change, invasive species, pollution, and overfishing have all shifted ocean conditions. Factors associated with climate change such as rising temperatures, increasing acidification, and changes in precipitation can influence marine diseases (Burge *et al*., 2014). Invasive species can increase diseases via the introduction of new parasites or acting as reservoirs for existing diseases (Chinchio *et al*., 2020).

Table 1 gives an overview of the main disease-causing agents present in gastropods ranging from viruses to macroparasites. Due to their commercial importance abalone have been some of the most studied marine gastropods with regards to their disease status. Very little information is available on potential diseases of slipper limpets.

Table 1.1 Summary of diseases and pathogens in marine gastropods

Disease	Parasite	Host	Location	References	Comments
			Viruses		
Abalone viral ganglioneuritis	Haliotid herpesvirus-1 (HaHV-1)	Abalone; e.g. Variously coloured abalone, *Haliotis diversicolor* (Reeve, 1846); Disk abalone, *H . discus* (Reeve, 1846); Blacklip abalone, *H. rubra* (Leach, 1814)	Taiwan, China, Australia	(Corbeil, 2020)	Clinical signs of infection include swollen mouth, prolapse odontophores, mantle recession, and muscle atrophy
			Bacteria		
Withering syndrome	*Candidatus* Xenohaliotis californiensis	Abalone e.g. *Haliotis cracherodii* (Leach, 1814), *H. rufescens* (Swainson, 1822), *H. sorenseni* (Bartsch, 1940)	Originated along the eastern pacific margin of California US, and Californica, Mexico, now has a broad geographical range	(Crosson *et al*., 2014)	Pedal and digestive gland atrophy, metaplasia of the digestive gland
Vibriosis	*Vibrio* spp. e.g., *Vibrio harveyi*	Range of hosts e.g., Abalone *Haliotis tuberculata* (Linnaeus, 1758); sea snail *Nassarius mutabilis* (Linnaeus, 1758), *Nerita albicilla* (Linnaeus, 1758)	Global	(Kumazawa *et al*., 1988; Pichon *et al*., 2013; Serratore *et al*., 2019)	Clinical signs of infection can vary with species type, can include white spots that consist of necrotic muscle fibres and bacteria on the foot on the Abalone *Haliotis diversicolor*
			Fungi		
	Atkinsiella awabi	Abalone, *Haliotis sieboldii* (Reeve, 1846)	Japan	(Kitancharoen *et al*., 1994)	Tubercle-like swelling on the mantle and melanized lesions on the peduncle
	Halipthoros milfordensis	Giant abalone, *Haliotis gigantea* (Gmelin, 1791)	Japan	(Hatai, 1982)	External symptoms include flat/tubercle-like swelling on mantle, epipode and dorsal surface of foot
	Halioticida noduliformans	South African abalone, *Haliotis midae* (Linnaeus, 1758)	South Africa	(Macey *et al*., 2011)	Clinical signs include areas of necrotic tissue or ulceration

	Microsporidium aplysiae	California sea hare, *Aplysia californica* (Linnaeus, 1758)	Northern Californica, USA to Gulf of Californica, Mexico	(Krauhs *et al*., 1979)	Does not appear to disturb host cells of *A. californica*
			Haplosporidia		
	Haplosporidium montforti	Abalone, *Haliotis tuberculata* (Linnaeus, 1758)	Galicia, NW Spain	(Azevedo *et al*., 2008)	Signs of infection include dark foot pigmentation, loss of surface adherence and an inability to right themselves. However, these signs are not always specific to infection with *H. montforti*
	Haplosporidium tuxtlensis	False limpet, *Siphonaria pectinate* (Linnaeus, 1758)	Gulf of Mexico	(Vea and Siddall, 2011)	No outwards sign of disease detected
	Haplosporidium lusitanicum	Blue rayed limpet, *Patella pellucida* (Linnaeus, 1758)	N. Portugal	(Azevedo, 1984)	No outwards sign of disease detected
	Haplosporidium patagon	Lesson's false limpet, *Siphonaria lessonii* (Blainville, 1827)	Patagonia, Argentina	(Ituarte *et al*., 2014)	No outwards sign of disease detected
			Apicomplexa		
	Cocidian spp.	Queen Conch, *Strombus gigas* (Linnaeus, 1758)	Colombia	(Richard *et al*., 2007)	Total invasion of every alveolar cell in an infected animal
	Digyalum oweni	Periwinkle, *Littorina obtusata* (Linnaeus, 1758)*; L. saxatilis* (Olivi, 1792)*; L. mariae* (Turton, 1825)*, L. arcana* (Hannaford-Ellis, 1978)	UK, Nova Scotia, Canada	(Dyson *et al*., 1992)	In *L. obtusata* some local tissue damage at the point of attachment has been observed e.g. missing cilia. No other sings of damage have been observed
	Merocystis kathae	Common whelk, *Buccinum undatum* (Linnaeus, 1758)	Northern Europe	(Dakin, 1911; Kristmundsson and Freeman, 2018)	Heavy infection characterized by numerous small white cysts on the kidney
	Nematopsis gigas	Sea snail, *Nerita ascencionis* (Gmelin, 1791)	Brazil	(Azevedo and Padovan, 2004)	Clinical sings not well described in gastropods. In the Indian oyster *Crassostrea madrasensis,* infection with *Nematopsis* sp. has been reported to cause compression of the digestive diverticula, and phagocytosed oocysts
Perkinsosis	*Perkinsus olseni*	Blacklip abalone, *Haliotis rubra* (Leach, 1814)*;* greenlip abalone, *H. laevigata*	Australia	(Lester and Davis, 1981; Bower, 2010)	Clinical signs include brown, sphere- shaped abscesses in the foot and mantle. Thin, watery tissue

		(Donovan, 1808); whirling abalone, *Haliotis cyclobates* (Peron and Lesueru, 1816); ridged ear abalone, *H. scalaris* (Leach, 1814)			with a pale digestive gland. Nodules in gills and mantle
			Paramyxids		
Marteiliosis (QX disease)	*Marteilia sydneyi*	Sydney rock oyster, *Saccostrea glomerata* (Gould, 1850)	Queensland, Australia	(Berthe *et al*., 2004)	Clinical signs include shrunken body, poor condition, colourless and translucent tissues, digestive gland become pale yellow-brown
Aber disease	*Marteilia refringens*	*Ostrea edulis* (Linnaeus, 1758), *Mytilus galloprovincialis* (Lamarck, 1819)	Atlantic coast of Europe from southern United Kingdom to Spain, and in the Mediterranean Sea	(Alfjorden *et al*., 2004)	Poor condition index, discoloration of digestive gland, cessation of growth, tissue necrosis (Bower, 2019)
			Polychaeta		
	Bristleworm, *Polydora cilata*	Slipper limpet, *C. fornicata* (Linnaeus, 1758)	Wadden Sea	(Thieltges *et al*., 2006a)	Heavy infestation in the mussel *Mytilus edulis* has been suggested to result in shell weaking (Kent, 1981)
	Polydora spp., *Dipolydora armata, Boccardia knoxi*	Abalone, e.g., *Haliotis kamtschatkana* (Jonas, 1845); *H. diversicolor* (Reeve, 1846), *H. midae* (Linnaeus, 1758), *H. iris* (Gmelin, 1791)	Global, e.g. Southern Japan, South Africa, Australia, British Columbia, China	(Bower, 2009a)	Flesh weight of *H. diversicolor* has been shown to decrease with heavy infestation (Kojima and Imajima, 1982). Shell damage and stress observed in *H. midae,* and *H. kamtschatkana* (see Simon *et al*. 2006)
			Digenea		
	Renciola sp., *Microphallus similis, M. pygmaeus, Hismasthla elongata*	Rough periwinkle, *Littorina saxatilis* (Olivi, 1792), *L. arcana* (Hannaford-Ellis, 1978); Common periwinkle, *Littorina littorea* (Linnaeus, 1758), Mud snail, *Hydrobia ulvae* (Pennant, 1777)	North Sea, Wadden Sea	(Thieltges *et al*., 2006b; Bojko *et al*., 2017)	Effects on host rarely reported. Some reports of an increase in spire height in *L. saxatilis* infected with *Microphallus piriformes* (see McCarthy *et al*. 2004)
	Allopodootyle sp.	Donkey ear abalone, *Haliotis asinine* (Linnaeus, 1758)	Heron Island Reef, Australia	(Lucas *et al*., 2005)	Parasitic castration can occur as a result of infestation (Rice *et al*., 2006)

			Nematoda		
	Echinocephalus pseudouncinatus	Pink abalone, *Haliotis corrugata* (Wood, 1828), Green abalone, *H. fulgens* (Philippi, 1845)	Southern California, USA, Gulf of California, Mexico	(Millemann, 1951; Bower, 2001)	Infestation can result in blister-like protrusions on the foot
			Cestoda		
	Parachristinaella sp., *Tylocephalum* sp., *Rhinebothrium* sp	*Crepidula fornicata* (Linnaeus, 1758), *Crassostrea virginica* (Gmelin, 1791), *C. gigas* (Thunberg, 1793)	Northern gulf of Mexico, Hawaii, Georgia, Japan, Taiwan, Australia	(Cake 1976; Bower 2009a)	Heavy infection can cause physiological stress and may affect growth and reproduction (Bower, 2009b)
			Turbellaria		
	Urastoma cyprinae	*Mytilus galloprovincialis* (Lamarck, 1819), *M. edulis* (Linnaeus, 1758), *Crassostrea virginica* (Gmelin, 1791), *Ostrea edulis* (Linnaeus, 1758), *Urastoma cyprinae* (Graff, 1882)	Europe, Gulf of Mexico, Atlantic Canada	(Fleming, 1986; Goggin and Cannon, 1989; Crespo-González *et al*., 2010)	Small oval or pyriform turbellarians up to 2 mm long present on gill surfaces and between gill lamellae
			Porifera		
	Cliona celata	Slipper limpet, *Crepidula fornicata* (Linnaeus, 1758); Common periwinkle, *Littorina littorea* (Linnaeus, 1758)	Widely distributed	(Stefaniak *et al*., 2005; Le Cam and Viard, 2011)	Production of extra shell material, through to no apparent detriment in *Crepidula fornicata.* Infestation in *Littorina littorea* has been show to result in a smaller body mass, and a weakened shell

1.4.1 Viruses in marine gastropods

Much of the knowledge regarding viruses in marine molluscs is restricted to those affecting farmed species such as oysters *Crassostrea gigas*, abalone *Haliotis diversicolor*, and the scallop *Chlamys farreri* (Müller, 1776; see Arzul *et al*. 2017) The viral family that has attracted the most attention in molluscs is the Malacoherpesviridae family. Ostreid herpesvirus 1 (OsHV-1) has resulted in mass mortalities of bivalve molluscs in Europe since 2008 (EFSA, 2010). Abalone herpesvirus (AbHV) is the causative agent for Abalone viral ganglioneuritis (AVG). AbHV has been assigned as the second member of the Malachoherpesviridia, after comparisons between AbHV and OsHV-1 showed similarities of 19-53% over some common coding regions (Savin *et al*., 2010). In 2005 there was an outbreak of AVG in Australian populations of *Haliotis laevigata* (Donovan, 1808) and *Haliotis rubra* (Savin *et al*., 2010). AVG resulted in up to 100% mortality in farmed abalone, on land-based farms in Victoria, Australia (Hooper *et al*., 2007). In 2006 the disease was found in wild abalone, initially in the area of the affected farms, and then spread along the Victorian coastline (Corbeil *et al*., 2012). A herpes-like virus was detected through electron microscopy in the nervous tissue of an infected abalone, and this was considered to be the pathogenic agent of the outbreak (Tan *et al*., 2008). A herpes-like virus was shown to be associated with high mortality rates in farmed *H. diversicolor* in Taiwan. Histology of moribund individuals showed that the nervous system was the primary tissue target for the virus. Lesions were characterized by tissue necrosis, alongside haemocyte infiltration (Pen *et al*., 2005). The aquaculture industry of *H. diversicolor* in southern China faced near collapse due to AbHV outbreaks since 1999 (Gu *et al*., 2019).

1.4.2 Bacteria

Withering syndrome

Withering syndrome is a fatal disease found in abalone caused by a Rickettsiales-like organism (Crosson *et al*., 2014). Rickettsiales-like organisms (RLOs) are intracellular bacteria that have been reported to be symbionts of more than 60 marine bivalve and gastropod molluscs (Cruz-Flores and Cáceres-Martínez, 2020). *Candidatus Xenohaliotis*

californiensis (*C*Xc; Murray and Schleifer 1994) is the only aetiological agent of withering syndrome in several abalone species (Friedman *et al*., 2000). *C*Xc occurs along the eastern Pacific margin of North America in California, USA, and Baja California, Mexico. Due to the transport of infected abalone to countries such as Chile, China, Spain, Ireland, and Thailand, the geographical extent is thought to be broad (Crosson *et al*., 2014). Withering syndrome infects the abalone gastro-intestinal epithelia and results in major morphological and functional abnormalities within the digestive gland (Crosson and Friedman, 2018). In abalone with withering syndrome, these effects to the digestive gland can cause physiological starvation, catabolism of the foot muscle, lethargy, and finally death (Friedman *et al*., 2002; Braid *et al*., 2005). Mortality in infected individuals appears to be species dependent. A mortality rate of up to 97% has been observed in the black abalone *Haliotis cracherodii* (Leach, 1814) in both laboratory and field studies in the USA (Altstatt *et al*., 1996), but no mortalities were observed in *Haliotis diversicolor* in Thailand (Wetchateng *et al*., 2010).

Vibriosis

Vibrio spp. are ubiquitous in aquatic environments (Möller *et al*., 2021). The genus *Vibrio* is composed of over 130 Gram-negative species (Ceccarelli *et al*., 2019). Coastal species can result in infections in humans, these species are an emerging health concern globally with links to climate change (Diner *et al*., 2020). Species that are of major concern for their impacts on human health include *Vibrio cholerae*, the causative agent of chlorea, that can be ingested through contaminated drinking water, and is responsible for 21,000-143,000 deaths globally each year (Ali *et al*., 2015). Other species of concern, are *Vibrio parahaemolyticus* and *V. vulnificus*, as ingestion of contaminated shellfish can cause wound infections, septicaemia, and gastroenteritis (Wang *et al*., 2015; Phillips and Satchell, 2017)

Vibriosis has been a major problem from gastropod culture globally, especially abalone, with several *Vibrio* spp. reported as the causative agents (Grandiosa *et al*., 2020). *Vibrio parahaemolyticus* was responsible for mass mortalities in cultured abalone *Haliotis diversicolor supertexta* (Lischke, 1870) in Taiwan and China (Lee *et al*., 2001; Cai

et al., 2007). Along the French coast, *Haliotis tuberculata (*Reeve, 1846) has been reported to be infected with *Vibrioharveyi* (Pichon *et al*., 2013). *Vibrio harveyi* resulted in the death of 60,000 cultured Ezo abalone *Haliotis discus hannai* within just a few days on a Japanese abalone farm in August 2002 (Sawabe *et al*., 2007). *Vibrio harveyi* and *V. splendidus* have been isolated from moribund blacklip abalone *H. rubra* (Leach, 1814) and greenlip abalone *H. laevigata* (Leach, 1814) during disease outbreaks in Tasmania, Australia (Handlinger *et al*., 2005). *Vibrio* spp. are thought to be opportunistic pathogens, as they can cause acute infection and death in physically or environmentally stressed individuals (Hooper et al., 2011, 2014). Increased mortality in *H. laevigata*, *H. rubra*, and *H. tuberculata*, has been associated with increased water temperatures with promote infection with *Vibrio* spp. such as *V. harveyi*, and *V. parahaemolyticus*(see Cheng *et al*. 2004; Vandepeer 2006; Travers *et al*. 2014).

1.4.3 Disease caused by fungi and oomycetes

Few cases of fungal infections in marine gastropods have been reported to date. In Japan, infections by the parasitic oomycete *Halipthoros milfordensis* (Vishniac, 1958) and the fungus *Atkinsiella awabi* sp. (Kitancharoen *et al*., 1994) have been reported in the giant abalone *Haliotis gigantea* (Gmelin, 1791; see Hatai 1982; Kitancharoen *et al*. 1994). External signs of disease included flat or tubercle-like swellings on the mantle, epipode, and dorsal surface of the foot. An outbreak of tubercle mycosis was reported in *H. midae* (Linnaeus 1758) culture farms in South Africa (Macey *et al*., 2011). Infected animals were characterized by necrosis of the epithelium, underlying muscle fibres and connective tissues of the foot, epipodium and mantle. The pathogen that was identified as causing this mycosis outbreak was the oomycete *Halioticida noduliformans* (see Muraosa *et al*. 2009; Macey *et al*. 2011). This outbreak had devastating effects on aquaculture production, resulting in up to 90% mortality in spat and 30% mortality in older animals.

Microsporidia are a large group of unicellular obligate intracellular spore forming eukaryote parasites (Han *et al*., 2020). They form over 50% of the described pathogenic

fungal species of marine animals, primary parasitizing arthropods and teleost fish (Pang *et al*., 2021). In molluscs, microsporidians have been described in bivalve species such as the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819), and the blue mussel *Mytilus edulis* (Linnaeus, 1758; see Comtet *et al*. 2004). The only reports of a microsporidian infecting a marine gastropod species is the case of *Microsporidium aplysiae* (Krauhs *et al*., 1979) in the gastropod *Aplysia californica* (Cooper, 1863; see Krauhs *et al*. 1979).

1.4.4 Haplosporidia

The phylum Haplosporidia contains a small group of histozoic or coelozoic, spore forming, endoparasitic protists (Burreson and Ford, 2004). The phylum contains four genera (*Haplosporidium* (Caullery & Mesnil, 1899), *Minchinia* (Lankester, 1885), *Urosporidium* (Caullery & Mesnil, 1905.), and *Bonamia* (Pichot *et al*., 1979) with over 52 described species and a number of unnamed species (Azevedo and Hine, 2017). Haplosporidia have a broad distribution in both marine and freshwater invertebrates (Hartikainen *et al*., 2014).

A handful of haplosporidians have been reported in marine gastropods e.g. *Haplosporidium lusitanicum* (Azevedo, 1984) in the limpet *Helicon pellucidus* (Linnaeus, 1758; see Azevedo 1984b), *H. montforti* in the European abalone *Haliotis tuberculata* (Linnaeus, 1758; see Azevedo *et al*. 2008), *H. tuxtlensis* in the false limpet *Siphonaria pectinate* (Linnaeus, 1758; see Vea and Siddall 2011), and *H. patagon* in *Siphonaria lessonii* (Blainville, 1827 see Ituarte *et al*. 2014). Mortalities of black-footed abalone *Haliotis iris* (Gmelin, 1791) occurred at a facility in New Zealand April 2000 (Diggles *et al*., 2002). The presence of the haplosporidians in the affected culture site was associated with mortalities of slow growing abalone during the summer months. Total mortalities reached between 80-90%. Individuals with high levels of infection showed abnormal behaviour, including lethargy, loss of righting reflex, and were easily detached from surfaces. Some individuals also showed swelling, and lesions in the foot and mantle (Diggles *et al*., 2002). Juvenile abalone *H. tuberculata* (Reeve, 1846) originally from Ireland and experimentally grown in plastic barrels suspended from rafts in Galicia

NW Spain, experienced mortality rates of 100% during spring and summer, the individuals were all heavily infected with *H. montforti* (see Azevedo *et al*. 2006)

1.4.5 Paramyxids

Paramyxida is an order of rhizarian protists that are known to parasitize marine molluscs, annelids and crustaceans (Ward *et al*., 2016). They include notifiable pathogens of bivalvies and other economically important species, with the most well known being *Marteilia* spp. (Ward *et al*., 2016). *Marteilia sydneyi,* the causative agent of QX (Queensland Unknown) disease, is recognised as the most severe parasite to infect the Sydney rock oyster, *Saccostrea glomerata* , on the east coast of Australia (Adlard and Nolan, 2015). Outbreaks of QX disease can result in >95% mortality (Bezemer *et al*., 2006). *Marteilia sydneyi* infection generally occurs in January-February and may be associated with heavy rainfall (Butt *et al*., 2006). Peters and Raftos (2003) have shown that *M. sydneyi* infects oysters after their immune system, notably the phenoloxidase cascade which is a key form of defence becomes supressed. Control measures typically require quarantining entire estuaries in order to limit the movement of infected stock.

Marteilia refringens infects commercially important bivalve species such as the flat oyster *Ostrea edulis* and mussels *Mytilus galloprovincialis* (Villalba *et al*., 1993; Robledo and Figueras, 1995; Le Roux *et al*., 2001; López-Flores *et al*., 2004; Novoa *et al*., 2005). *Marteilia refringens* has been recorded in Europe from the northern coast of France, to the Mediterranean Sea, Corsica, Italy, Slovenia, Portugal, Croatia, Greece and Tunisia, and has been responsible for repeated mass mortalities of *Ostrea edulis* over the last 40 years (reviewed by Berthe *et al*. 2004). The World Organization for Animal Health (OIE) and the European Union (under EC Directive 2006/88) has recognised it as a significant pathogen of bivalve molluscs (OIE, 2017).

1.4.6 Apicomplexa

Apicomplexans are a group of unicellular, often pathogenic, obligate parasites, that exploit either one or two hosts to complete a full reproductive cycle (Kristmundsson and Freeman, 2018). The most known apicomplexans include *Plasmodium* (Golgi, 1886)

that causes malaria, and *Toxoplasma* (Nicolle & Manceaux, 1909) that results in toxoplasmosis (Votýpka *et al*., 2017). Apicomplexans can also infect marine species. Those present in the marine environment include *Perkinsus olseni* (Lester & Davies, 1981) in abalone *H. rubra, H. laevigata, H. cyclobates* (Péron & Lesueur, 1816) and *H. scalaris* (Leach, 1814) along the coast of South Australia (Bower, 2010). *Merocystis kathae* (Dakin, 1911) has been found in the common whelk *Buccinum undatum* (Linnaeus, 1758) from the Irish Sea (Dakin, 1911). *Digyalum oweni* (Koura *et al*., 1990) is mainly found to infect the common flat periwinkle *Littorina obtusata* (Linnaeus, 1758), but has been identified in all Atlantic *Littorina* (Linnaeus, 1758) species including *L. saxatilis* (Olivi, 1792) and *L. arcana* (Hannaford-Ellis, 1978), though the presence of the parasite does not appear to harm the host (Dyson *et al*., 1992). The apicomplexan *Nematopsis gigas* (Schneider, 1892) has been recorded in the mantle tissues of the gastropod *Nerita ascencionis* collected in the Northeast coast of Brazil (Azevedo and Padovan, 2004). An atypical reproduction cycle in the queen conch *Strombus gigas* was observed in a Columbian population with mature stages of gametogenesis observed in less than 10% of the population; infection with the apicomplexan coccidian was suspected to be the cause (Richard *et al*., 2007). Azmi *et al*. (2019) carried out a survey on a number of gastropod species including the conchs *Laevistrombus canarium (*Linnaeus, 1758) and *Canarium urceus* (Linnaeus, 1758), in the Merambong seagrass bed in Johor Straits, Malaysia, and found a high rate of infestation by apicomplexan parasites such as *Pseudoklossia* (Léger & Duboscq, 1915), and *Nematopsis* (de Beauchamp, 1910).

1.4.7 Macroparasites

Intertidal molluscs such as gastropods and bivalves are known to contain a rich diversity of macroparasites (de Montaudouin *et al*., 2000; Prinz *et al*., 2010). A survey a tidal basin in the Wadden Sea carried out by Thieltges *et al.* (2006) found 31 different macroparasite species in 3,800 host individuals from 10 host species. The gastropods *Hydrobia ulvae* (Pennant, 1777) and *Littorina littorea,* and bivalves *Cerastoderma edule* and *Mytilus edulis,* had the highest parasite burdens.

Digenea

Digenea are parasitic Platyhelminthes that have a wide distribution and, as a parasitic group, are known to infect the largest diversity of animals (Esch *et al*., 2002). These parasites can have a large impact on the tissue structure and morphology due to their size relative to their host (Mahony *et al*., 2021). Digenetic trematodes often infect 2-3 hosts in order to complete their life cycle, with a gastropod most commonly serving as a first intermediate host (Blakeslee *et al*., 2020) (Figure 1.3).

Figure 1.3 Life cycle *of Microphallus similis* (Jägerskiöld, 1900) in eastern North America with *Carcinus maenas* (Linnaeus, 1758) as second intermediate host. *Microphallus similis* infects multiple hosts in order to complete its life cycle, beginning with (A) two *Littorina* snail species (*L. saxatilis* and *L. obtusta*), where the trematode reproduces asexually, producing many cercariae. (B) The snail sheds the cercariae into the water column, where they seek out and encyst as a metacercariae inside a second-intermediate host, primarily the green crab *Carcinus maenas*. For sexual reproduction to occur, (C) the trematode's crab host needs to be ingested by a definitive host, often a *Larus* species, where (D) the trematode's eggs, containing miracidia, are subsequently deposited into the marine environment via the birds' faeces. These eggs are then accidentally consumed by grazing snails, continuing the life cycle (Figure from Blakeslee *et al*. 2015).

Once in the first intermediate host, trematodes go through asexual reproduction, subsequently shedding cercariae that may infect their definitive host. The host may become infected through direct penetration e.g. avian schistosomes, or more frequently, by encysting in a second intermediate host, that is eaten as prey by the definitive host (Lambert *et al*., 2016). Gastropods that are known to act as first intermediate hosts include *L. littorea*, which is the first host for *Cryptocotyle lingua*

(Creplin, 1825) and *Cercaria parvicaudata* (Stunkard & Shaw, 1931). *Littorina littorea* consumes the eggs of both parasites that have been deposited in the faeces of gulls, the final host. Cercariae are released from infected *Littorina littorea* and can infect a second intermediate host e.g., *Cercaria parvicaudata* infects bivalve molluscs such as *Mytilus edulis*. Transmission to the final host occurs through the consumption of the second intermediate host (Galaktionov and Dobrovolskij, 2003). In some incidences, gastropods can also act as the second intermediate host when cercaria remain in the first intermediate host, and develop into metacercariae e.g. *Microphallus pygmaeus* (Levinsen, 1881) and *Psilochasmus aglyptorchis* (Loos-Frank, 1968; see Thieltges *et al*. 2006). Trematode species such as *Renicola* sp (Cohn, 1904), and *Microphallus similis* (Jägerskiöld, 1900) have been recorded in *L. saxatilis* (Jamie Bojko *et al*., 2017). The digenean trematode *Allpodoctyle* sp (Pritchard, 1966). has been known to cause parasitic castration in the abalone *Haliotis asinina* (Linnaeus, 1758). This castration occurs because of alterations to the expression of a range of host neuropeptide genes involved in the control of gonadogenesis and not the direct consumption of the gonad by the trematode (Rice *et al*., 2006).

Cestoda

Cestoda, or tapeworms, form a large class of the Plathyhelminths with about 8000 species (Dezfuli *et al*., 2021). Cestodes are hermaphrodites, but self-fertilization is not common. Therefore, reproduction generally occurs through cross-fertilization, requiring additional organism for the continuation of the species within the host (Apeti *et al*., 2014). The life cycle of cestodes requires one intermediate host (e.g. bivalves), which harbour eggs and larvae (encysted sporocysts and metacercariae larvae), and one definitive host (vertebrates) which harbour the adult worms (Roberts *et al*., 2005). In molluscs, the host response to being infected is characterized by encapsulation of larval cestodes in connective tissue, with little harm often occurring (Apeti *et al*., 2014). Cestodes such as *Parachristianella* sp., *Tylocephalum* sp., and *Rhinebothrium* sp., have been recovered from *Crepidula fornicata* collected from the northern gulf of Mexico during a study by Cake (1976).

Turbellaria

Turbellarians are generally accepted as being hermaphroditic, free-living Platyhemmintehes (Mladineo *et al*., 2012). Turbellarians have been recorded in a wide range of molluscs globally, ranging from *Cerastoderma edule, Mytilus galloprovincialis,* and *Ostrea edulis* in Europe (Robledo *et al*., 1994; Aguirre-Macedo and Kennedy, 1999; Carballal *et al*., 2005) to the Pacific geoduck (*Panopea generosa;* Gould, 1850) in Mexico (Cáceres-Martínez *et al*., 2015). Turbellarians have been known to act in both an endocommensal and parasitic nature in molluscs (Shanks, 2001) and have the capacity to move between the mantle cavity and alimentary canal of its host (Longshaw and Malham, 2013). In general turbellarians are found in low prevalence and intensities and often appear to cause little to no harm to its host, very few clinical signs of infection are apparent with the potential exception of the appearance of white to pink coloured "gill worms" up to 2 mm long on gill and mantle surfaces (Meyers and Burton, 2009).

Nematoda

Very few observations have been made regarding the presence of nematodes in marine gastropods. *Echinocephalus pseudouncinatus (*Millemann, 1951) has been described in the abalones *Haliotis corrugata* (Wood, 1828.) and *H. fulgens* (Philippi, 1845; see Millemann, 1951). Abalone and sea urchins act as intermediate hosts for *E. pseudouncinatus,* with elasmobranch fish such as the horned shark *Heterodontus francisci* (Girard, 1855) acting as the definite host (Millemann, 1963). Clinical signs of infection with *E. pseudouncinatus* include blister-like cysts in the ventral portion of the foot containing larval nematodes (Bower, 2001).

Porifera

Le Cam and Viard (2011) studied the temporal prevalence of the shell boring sponge *Cliona celata* (Grant, 1826) on *C. fornicata. Crepidula fornicata* was found to produce extra shell material when infested with the endolithic sponge. Sponges in the Clionidae family generally live in a network of tunnels and cavities that they create by excavating the calcium carbonate substrates found in the shells and skeletons of living molluscs and corals (Stefaniak *et al*., 2005). The sponge does not directly feed on the host tissue,

however boring can have a range of negative impacts on hosts, including an increase chance of skeletal breakage and/or direct and indirect costs of shell repairs.

In molluscs, shells act as protection from predators, and shell thickness and strength have a direct impact on an individual's vulnerability to shell- breaking predators such as lobsters and crabs (Kitching and Lockwood, 1974). Boring by sponges such as *C. celata* can weaken shells, making them more susceptible to predation. Molluscs such as *Crepidula fornicata* respond to boring by laying down more shell material (Le Cam and Viard, 2011). Shell production is energetically costly and reduces the available energy for somatic growth and reproduction (Palmer, 1981). Increasing material on the interior of the shell can limit snail living space resulting in a potential decrease in body size and fecundity (Hughes and Roberts, 1980). *Littorina littorea* in the southern Gulf of Maine has also been found to be infested with *Cliona* sp. The host was found to have a reduced interior shell volume, lower dry body mass, and a weakened shell when infested with *Cliona* sp. (Stefaniak *et al*., 2005).

1.4.8 Parasites and pathogens of *Crepidula fornicata*

Presently, little information is available on the disease status of *Crepidula fornicata.* The most common pest to affect *Crepidula fornicata* is the shell boring sponge *C. celata* (see Le Cam and Viard 2011). A small-scale disease survey for the presence of trematodes in *Crepidula fornicata* found that they were not acting as an intermediate host, both in their native population, and at invaded sites (Pechenik *et al*., 2001; Thieltges *et al*., 2006a). Pechenik *et al*. (2012) studied the closely related species *Crepidula plana* (Say, 1822) and *Crepidula convexa* (Say, 1822) from Rhode Island and Massachusetts, U.S.A. are found no sign of parasitic infection. Finally, Thieltges *et al*. (2006b) found only one parasite species in *C. fornicata,* the annelid worm, *Polydora ciliata*, during a screen of intertidal molluscs in the Wadden Sea.

1.5 Gastropod immunobiology

Gastropods inhabit both aquatic and terrestrial environments, encountering a wide range of parasites ranging from microscopic viruses such as abalone viral ganglioneuritis (AVG) to the macroscopic parasites such as digenean trematodes (Strong *et al*., 2008; Prinz *et al*., 2010; Nicolai and Ansart, 2017; Corbeil, 2020). Though initial thought to possess an unsophisticated innate immune system in order to protect from foreign particles and organisms, it is now known that gastropods and other invertebrates can deploy an immune response that can be incredibly complex, diverse, and can be specific to the parasite that they are defending against (Loker *et al*., 2004; Ghosh *et al*., 2011; Deleury *et al*., 2012; Dheilly *et al*., 2014; Zhang *et al*., 2014). Much of the knowledge gained around gastropod immunobiology has come about from studies focused on the commercially important marine abalone *Haliotis* spp. and the freshwater snail *Biomphalaria* spp. due to it being the intermediate host for the parasitic disease-causing trematode *Schistosoma* (see Cardinaud *et al*. 2015; Coustau *et al*. 2015; Pila *et al*. 2017; You *et al*. 2019; de Melo *et al*. 2020).

1.5.1 External barriers

Exoskeletons have independently evolved 18 times over 550 million years and have aided in the success of the Gastropoda (Rae, 2017). Gastropod shells consist of an outer proteinaceous periostracum of conchiolin and sub-layers of crystalline calcium carbonate (Rae, 2017). The shell aids in protection from predators and extreme environmental conditions. However, it has been reported that gastropod shells can also be used as a defence system to encase and kill parasitic nematodes. In a study carried out by Rae (2017), it has been demonstrated that upon infection, cells on the inner layer of the shell stuck to the nematode cuticle, swarm over its body and fuse it to the inside of the shell. Shells of wild *Capaea nemoralis* (Linnaeus, 1758), *C. hortensis* (Müller, 1774) and *Cornu aspersum* (Müller, 1774) have been seen to be heavily infected with a number of nematode species. A ciliated, mucus-producing epithelium protects the soft, moist body surfaces of gastropods. This epithelium acts as a physical trap and barrier to colonization by pathogens (Loker, 2010). The regular production and shedding of

body mucus may also be advantageous in allowing for the cleansing of surfaces of pathogens like bacteria which are stimulated to grow in biofilms in mucus trails. Guo *et al*. (2009) studied the effects of small abalone, *H. diversicolor*, pedal mucus on bacterial growth, attachment, biofilm formation and community structure. Results showed that pedal mucus enhanced the growth of bacteria, and that more opportunistic pathogens e.g. *V. alginolyticus* attached to a substrate covered with pedal mucus. If these external barriers are no longer offering protection from parasites and pathogens, then the internal defences involving cellular and humoral (soluble) components are used. The cellular component consists of circulating haemocytes that perform tasks such as phagocytosis and encapsulation, and followed by the destruction of the pathogen through enzyme activity and release of oxygen metabolites (Wang *et al*., 2018). Humoral components of the molluscan immune system consist of nitric oxide, lysozyme activity, lectins, and the phenoloxidase system (Al-Khalaifah and Al-Nasser, 2019).

1.5.2 Pathogen recognition

The ability to discriminate between self and non-self is central to the immune system of all organisms (Buchmann, 2014). In gastropods and other invertebrates, innate immune receptors called PRRs (Pattern-Recognition Receptors) trigger immune signalling pathways (Tetreau *et al*., 2017). There are four main sub-groups of PRRs i.e. the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain-like receptors (NLR), RIG-like receptors (RLRs) and C-type lectin receptors (CLRs)(Walsh *et al*., 2013). PRRs identify pathogen-associated molecular patterns (PAMPS), and trigger an immune response in order to eliminate any pathogens (Sánchez-Salgado *et al*., 2021). Genes encoding TLRs have been described in gastropod species such as the common periwinkle *L. littorea* and the disk abalone *H. d. discus* (see Elvitigala *et al*. 2013; Gorbushin 2020). A C-type lectin has been described in the Pacific abalone *H. discus hannai* (Zhang *et al*., 2014).

1.5.3 Cellular defences

Gastropods possess an open circulatory system which is populated with haemocytes. With *Crepidula fornicata* possessing only one type of haemocyte. These form the cellular component of haemolymph but can also be found in other sites such as the connective and vascular tissues (Loker, 2010). Haemocytes are a key factor in the molluscan innate immune system (Melillo *et al*., 2018). Phagocytosis, multicellular encapsulation and cell-mediated cytotoxicity, are the currently known effector mechanisms of haemocytes (Lange *et al*., 2017). Gastropods can also possess "fixed" defensive cells that are found around internal spaces and trap and/or phagocytose particulate objects (Matricon-Gondran and Letocart, 1999). Besides their defensive role, haemocytes also take part in wound healing, nerve repair, shell formation and repair, tissue remodelling potentially including the dissolution of gonads after spawning, and the movement of metabolites and nutrients (Loker, 2010). Gastropod haemocytes also take part in diapedesis i.e., the movement of ingested foreign material to and across external or gut epithelia (Gust *et al*., 2011).

Many of these roles result in the depletion of circulating haemocytes, which must be replenished by haematopoiesis. The normal turnover of haemocytes through migration across external epithelia, and senescence also require replacement (Pila *et al*., 2016). The location of this regeneration varies massively. In a number of gastropods, haemocyte production takes place in the pericardial region or in anatomically similar structures (Sullivan, 1988; Ottaviani, 2006). Other reported potential areas of haematopoiesis in gastropods include the external surface of the ctenidial/pulmonary and renal veins in the pericardial cavity (Accorsi *et al*., 2014). In many molluscs, a haematopoietic site has yet to be described including in *C. fornicata.*

Gastropod haemolymph does not typically coagulate when removed from the animal or post exposure to pathogens (Loker, 2010). Tubular helical filaments visible via electron microscopy have been observed in haemolymph of gastropod species such as the California sea hare *Aplysia californica* (Cooper, 1863), and the Giant keyhole limpet

Megathura crenulata (Sowerby, 1825; see Harris and Markl 1992; Martin *et al*. 2007), and plasma of the snail *Biomphalaria glabrata* (Say, 1818) infected with the trematode *Echinostoma caproni* (Richard, 1964) regularly forms precipitates (Wu *et al*., 2017). In comparison to other invertebrates, gastropod haemocytes have comparatively few distinct morphological features, generally lacking large numbers of visible cytoplasmic granules or pigments, lack flagella, and do not have the clear tendency to lyse when removed from the host or faced with an antigenic stimuli (Loker, 2010). A tendency observed more so in marine than terrestrial gastropods is for haemocytes to readily form aggregates (Gorbushin and Iakovleva, 2006).

1.5.4 Humoral defences

The humoral immune response in gastropods is comprised of a diverse group of soluble molecules that are secreted in the haemolymph of animals, and have the capacity to be distributed systemically (Castillo *et al*., 2015). Humoral components include lysozymes, reactive oxygen intermediates (ROIs) and the phenoloxidase system. Lysozymes are a diverse group of proteases that are capable of hydrolysing amino-glycosidic bonds such as those found in the peptidoglycan layers of the surface of prokaryotes (Callewaert and Michiels, 2010). The role of lysozymes in defence and inflammation has been describe in gastropods such as the disk abalone *H. d. discus* (see see Bathige *et al*. 2013). Reactive oxygen species such as superoxide anion (O^{-}_{2}) have an important role in microbicidal activity in gastropods (Cheng *et al*., 2004).

1.5.4.1 The prophenoloxidase system

A crucial defence strategy of invertebrates is the use of the enzyme, phenoloxidase (PO) in the haemolymph and solid tissues to induce melanin synthesis and the formation of other immune molecules (Smith and Soderhall, 1991; Cerenius *et al*., 2008). POs are present in the haemolymph in their inactive form (proPO) and are activated by external microbial components such as ß-1,3-glucans and lipopolysaccharide (LPS) (Söderhäll and Cerenius, 1998; Phupet *et al*., 2018). These components result in the

cleavage of proPO into PO by endogenous serine proteases (Cerenius *et al*., 2010b). PO then catalyses the hydroxylation of monophenols such as tyrosine to *o*-diphenol (DOPA or DOPAmine), followed by their oxidation to *o*- quinones (DOPAquinone and DOPAminequinone). Finally, the pigment melanin is generated at the end of the cascade. The first study to indicate the importance of PO in molluscs was carried out by Peters and Raftos (2003), who found that it was suppressed in Sydney rock oysters *Saccostrea glomerata* (Gould, 1850) from areas prone to QX disease when compared to those from QX free areas. A strong negative correlation between PO activity and the intensity of parasitic infection was also observed. The data suggested that the inhibition of the prophenoloxidase cascade could result in lethal infection by the aetiological agent of QX disease, the protozoan paramyxid, *Marteilia sydneyi* (Perkins and Wolf, 1976; see Peters and Raftos 2003).

Studies of PO activity in marine gastropods appear to be limited to the commercially important abalone *Haliotis tuberculata* (Linnaeus, 1758; see Le Bris *et al*. 2014). The study concluded that this activity in *H. tuberculata* was mainly due to laccase-type activity. Along the French coastline, *H. tuberculata* was affected by mass mortality events as a result of both immune depression and bacteria *V. harveyi*. During these periods of immune depression, PO activity was monitored and was found to be decreased (Travers *et al*., 2008). Phenoloxidase has also been characterized in the freshwater gastropod *Biomphalaria* (Say, 1818; see Le Clec'h *et al*. 2016). *Biomphalaria* is a well-studied species due to it acting as the intermediate host of the blood fluke *Schistosoma mansoni* (Sambon, 1907), which infects over 250 million people globally (Mukendi *et al*., 2021). Le Clec'h *et al*. (2016) also determined that the phenoloxidase activity in *Biomphalaria* was a laccase-type activity.

Phenoloxidases can fall into one of three groups defined by their substrate specificity. Tyrosinases catalyse hydroxylation of monophenols and into *o*-diphenols and the oxidation of *o*-diphenols into *o*-quinones, catecholoxidase oxidize *o*-diphenols, and laccases oxidize *o*-diphenols, *p*-diphenols and *p*-diamines (Reiss *et al*., 2013; Whitten and Coates, 2017). In previous studies in PO research, L-3,4-dihydroxyphenylalanine (L-

DOPA) has been the primary substrate used. However, due to PO catalysing different reactions. Espín and Wichers (1999) developed a method for the determination of monophenolase activity, which should distinguish PO from other phenol-oxidizing enzymes, such as laccase, and catecholoxidase.

1.6 Aims of this thesis

The overall aim of this thesis was to investigate the disease harbouring potential of the invasive slipper limpet *C. fornicata,* and to gain an understanding of its haemolymph biochemistry. The thesis also sought to investigate potential "at risk" areas for invasion by *C. fornicata,* and how these areas could change under future predicted climate change. The following text summarises how the research was undertaken and reported in this thesis:

Chapter 2: Aimed to address the lack of knowledge with regards to the haemolymph biochemistry of *C. fornicata.* In this, haemolymph of *C. fornicata was* screened for the presence of the key immune enzyme, phenoloxidase.

Chapter 3: Investigated the role of *C. fornicata as* a sink for the Vibrio-like bacteria and whether vibriosis is an important disease of slipper limpets.

Chapter 4: A disease screen of *C. fornicata was* carried out to determine the presence or absence of haplosporidians, microsporidians, and paramyxids using a PCR and histological based method. *Crepidula fornicata were* also screened for the presence of macroparasites using whole-tissue histology.

Chapter 5: Aimed to construct a Species Distribution Model (SDM) to generate maps of the potential geographical distribution of *C. fornicata under* current climate conditions and to identify the key environmental factors affecting its current distribution. The SDM also aimed to investigate the potential future distribution of *C. fornicata under* predicted climate change scenarios.

Chapter 2 Exploring phenoloxidase-like activities in the haemolymph of the invasive slipper limpet Crepidula fornicata

A version of this chapter has been published:

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*SH Malkin and AF Rowley (co-supervisor) assisted with histology

2.1 Abstract

The slipper limpet *Crepidula fornicata* is an invasive, alien, marine species found throughout the coastal waters of southern England and Wales, UK. These limpets are considered to blight commercial shellfish banks, notably oysters, yet little is known about their disease-carrying capacity or their immunobiology. To address the latter, haemolymph (blood) was isolated from limpets and tested for the presence of the immune-enzyme phenoloxidase. Invertebrate phenoloxidases produce melanic polymers from simple phenolic substrates, which are deployed in the presence of pathogens because of their potent microbicidal and microbiostatic properties. A series of established substrates (e.g., tyrosine, hydroquinone) and inhibitors (e.g., 4-hexylresorcinol, benzoic acid) were used to target three distinct enzymes: laccase (*para*-diphenoloxidase), catecholoxidase (*ortho*-diphenoloxidase) and tyrosinase (monophenoloxidase). Laccase and catecholoxidase activities were confirmed and their kinetic properties across temperature and pH gradients (5–70 °C and 5–10, respectively) were characterised. Crucially, this chapter demonstrated that products derived from such laccase and catecholoxidase activities significantly reduced the numbers of colony-forming units of both Gram-positive and Gram-negative bacteria *in vitro.* Additionally, limpet tissues were screened for signs of melanin using wax histology, and cells replete with eumelanin-like pigments and lipofuscin in the digestive gland, connective tissues, barrier epithelia and gills were identified. This chapter represents the first account of enzyme-based antibacterial defences, notably laccase, in *C. fornicata.*

2.2 Introduction

An indispensable innate immune defence strategy of invertebrates is the use of phenoloxidase (PO) enzymes in the haemolymph and solid tissues to trigger melanin synthesis (Smith and Soderhall, 1991; Cerenius *et al*., 2008). The catalytic steps involved in converting simple phenolic substrates (e.g., tyrosine, dopamine) into pigment precursors (quinones), and ultimately melanin, generate antimicrobial by-products in the form of reactive oxygen/nitrogen species as well as semi-quinone intermediates (Zhao *et al*., 2007; Cerenius *et al*., 2010a; Zhao *et al*., 2011; Coates and Talbot, 2018). Often, the term phenoloxidase (PO) is used interchangeably to represent several distinct copper-containing enzymes: tyrosinase (EC 1.14.18.1), catecholoxidase (EC 1.10.3.1) and laccase (EC 1.10.3.2). Substrate and inhibitor specificities can be employed to discriminate between these phenoloxidases (POs). Tyrosinase catalyses the ortho-hydroxylation of monophenols (e.g., l-tyrosine) into ortho-diphenols (e.g., L-DOPA), and the two-electron oxidation of o-diphenols into o-quinones (e.g., DOPAchrome). Catecholoxidase performs the second reaction only, whereas laccase carries out the single-electron oxidation of both ortho and para-diphenols amongst other substrates (e.g., para-diamines; Reiss *et al*. 2013; Whitten and Coates 2017). The differences in catalysis can be attributed to their active sites; laccase contains a mononuclear (type1) copper site as well as a trinuclear copper cluster, whereas tyrosinase and catecholoxidase contain a dinuclear (type 3) copper site (Solomon *et al*., 2014). Such structural features of laccase facilitate its wide catalytic potential.

Once pathogens breach the physical barriers of the exoskeleton or integument, they are recognised in the haemolymph by circulating haemocytes equipped with pathogen recognition receptors that stimulate the proteolytic, prophenoloxidase activation cascade amongst other acute phase effectors (Cerenius *et al*., 2010b). Melanic polymers are generated and used to immobilise pathogens and facilitate their destruction – usually in concert with haemocyte encapsulation and nodulation.

Beyond innate immunity, phenoloxidases contribute to developmental morphogenesis, cuticle hardening and sclerotization post-ecdysis, and assist in clot development at wound sites (haemostasis; Bidla *et al*. 2009; Eleftherianos and Revenis 2011). Melanin-mediated defences have been studied extensively in insects (reviewed by González-Santoyo and Córdoba-Aguilar 2012) crustaceans (reviewed by Cerenius *et al*. 2008) and to a lesser extent, bivalves (Zhou *et al*. 2012; reviewed by Luna-Acosta *et al*. 2017). Conversely, such experimental evidence for a proPO cascade or tyrosinase is lacking for gastropods – an exception being the well- characterised (inducible) phenoloxidase activity of the oxygen-transport protein haemocyanin (Siddiqui *et al*., 2006; Dolashki *et al*., 2011; Raynova *et al*., 2013; Coates and Nairn, 2014; Coates and Costa-Paiva, 2020). Like the vast majority of invertebrates studied thus far, the gastropod innate immune repertoire consists of physical barriers (exoskeleton), cellular (haemocyte) and humoral (soluble) defences (Loker, 2010). To the best of our knowledge, in-depth biochemical characterisations of gastropod phenoloxidase(s) have been performed on the commercially important abalone genus *Haliotis* (see Le Bris *et al*. 2014) and medically important snail genus *Biomphalaria* (see Le Clec'h *et al*. 2016). In both instances, laccase-type phenoloxidase was the dominant form of activity recorded.

The slipper limpet *Crepidula fornicata* (Linnaeus, 1758) is an invasive, alien, marine gastropod in the Calyptraeidae family. It is native to the east coast of the United States of America but is now a pertinent example of an introduced species that can influence its non-native range (Orton, 1926; Cole and Baird, 1953; McNeill *et al*., 2010; Bohn *et al*., 2012). Slipper limpets were introduced accidently to European coastal waters at the end of the 19th century, most likely with shipments of *Crassostrea virginica* (Gmelin, 1791) being imported for the establishment of aquaculture (Blanchard, 1997). These limpets can be found in large numbers in most oyster production areas in England and Wales and are implicated in having a major negative impact on native bivalves, especially the European flat oyster *Ostrea edulis* (Linnaeus, 1758; see Hayer *et al*. 2019). In shallow bays, *C. fornicata can* smother the sediment forming beds with several

thousand individuals per m^2. Dense populations of *C. fornicata can* trap suspended silt, faeces and pseudo- faeces altering the composition and structure of the seabed (Chauvaud *et al*., 2000). Despite the sizeable volume of literature dedicated to the ecology of slipper limpets and their interactions with shellfish of commercial value, there remains a paucity of knowledge on their disease profiles, immunobiology or haemolymph biochemistry.

The overall aim of this chapter was to examine the haemolymph of *C. fornicata* for the presence of the immune enzyme, phenoloxidase. First, a combination of general and specific substrates and inhibitors were used to discriminate between putative phenoloxidases (monophenolase, *para-* and *ortho*-diphenolase). Second, the antiseptic properties of enzyme-catalysed reaction products were assessed in the presence of Gram-positive/negative bacteria, and third, limpet tissues were inspected for evidence of melanin using a histological approach.

2.3 Materials and Methods

2.3.1 Experimental Animals

Field sampling and collection of live adult *C. fornicata stacks* (Figure 2.1A) took place in the low intertidal zone (~0.8–1.5 m above chart datum) at Mumbles Beach, Swansea, South Wales, UK (51.571882, −3.987040). Samples were returned to the laboratory and processed immediately. Individuals were separated from stacks and cleaned of epibionts.

Figure 2.1 Typical stack formation of *Crepidula fornicata* (A) and accessibility of haemolymph after (solid) tissue removal (B). Black arrow points to pooled haemolymph at the aperture of the shell.

2.3.2 Isolation and preparation of haemolymph

Haemolymph was isolated from the animals by first removing the tissue mass from the shell using a blunt-ended probe and allowing the haemolymph to pool in the shell cavity (Figure 2.1B). The haemolymph was collected using a 22-gauge hypodermic needle fitted to a 1 mL sterile syringe. Haemolymph samples were combined from 3 to 5 limpets per replicate and centrifuged at 1000×*g* for 5 min at 4 °C to separate the haemocyte fraction. The cell-free supernatant was retained, stored at 4 °C, and used in enzyme assays within 1–2 days (no deterioration was observed for this duration).

2.3.3 Protein determination of the haemolymph

The total protein content of the *C. fornicata* acellular fraction of haemolymph was quantitated by the Biuret method (Gornall *et al*., 1949) using egg albumin (0– 20 mg mL^{-1}) as a protein standard.

2.3.4 Assay for phenoloxidase-like activities

Phenoloxidase activities were assayed spectrophometrically in 96-well microplates (Greiner 96-F-bottom) or 1 mL cuvettes using a BMG LABTECH SPECTROstar Nano equipped with a cuvette port and microplate reader. Each assay consisted of 100 mM sodium phosphate (NaPi) buffer pH7.4 and 1 mg mL^{-1} haemolymph protein (pre-incubated at room temperature (~20 °C) for 5 min). Substrates were added at varying concentrations (listed in Table 2.1) to initiate the reaction and run for 10 min (initial assays with representatives from all substrate types were run for 40 min, but rates of product accumulation slowed after 10 min). All assays were performed in triplicate (three technical replicates per biological replicate) at 20 °C. Results were systematically corrected for non-enzymatic autoxidation of each substrate in the absence of cell-free haemolymph. Enzymatic activities were recorded and converted to units [U: µmol per minute per mg (protein)] using the following absorption coefficients and wavelengths: 36,000 M^{-1} cm^{-1} for $ABTS^{+}$ (oxidised ABTS, A420 nm), 65,000 M^{-1} cm^{-1} for syringaldazine^{+} (oxidised syringaldazine, A525 nm), 1370 M^{-1} cm^{-1} for benzoquinone (oxidised hydroquinone, A390 nm), 1910 M^{-1} cm^{-1} for PPD^{+} (oxidised *p*-phenylenediamine, A520 nm), and 3600 M^{-1} cm^{-1} for DOPAchrome and its derivatives (oxidised $_{L}$-DOPA, dopamine and caffeic acid, A492 nm).

Table 2.1 Substrate parameters used to discriminate between phenoloxidase activities

Specificity	**Substrate**	**Molecular weight**	**Concentration range (mM)**	**Wavelength** [product detection]
Laccase	PPD	108.14	0.1-50	520
	Syringaldazine	360.36	0.005-10	525
	ABTS	548.58	0.01-15	420
	Hydroquinone	110.11	0.1-100	390
Non- specific	Caffeic acid	180.16	1-15	492
	L-DOPA	197.19	01.-30	492
	DHPPA	182.17	0.25-80	492
	Dopamine	189.64	0.1-30	492
Tyrosinase	L-Tyrosine	181.19	0.01-25	492
	4-HA	124.14	0.1-30	492
	Tyramine	137.18	0.1-30	492

PPD - para-Phenylenediamine
ABTS - 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
L-DOPA - 3,4-Dihydroxyphenylalanine
DHPPA -3,5-Dihydroxyphenylpropionoicacid
4-HA - 4-hydroxyanisole

2.3.5 Inhibition of phenoloxidase-like activities

Assays were prepared as described above, however, haemolymph protein (1 mg mL^{-1}) was pre-incubated with an inhibitor for 5 min prior to the addition of substrate, either hydroquinone (5 mM) or dopamine (5 mM). The inhibitors benzoic acid, citric acid, cetrimonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), 4-hexylresorcinol (4-HR), and phenylthiourea (PTU) were used across the concentration range 0.1–1 mM. Each combination of substrate and inhibitor was carried out in triplicate on three independent occasions. Inhibition data are expressed as the percentage reduction in enzymatic activity when compared to control values (i.e., substrate only).

2.3.6 Influence of pH and temperature on phenoloxidase-like activities

Assay mixtures were prepared as stated above, with 1 mg mL^{-1} protein, 5 mM of substrate (ABTS, dopamine or hydroquinone) in NaPi pH7.4, and incubated at 20 °C for 10 min prior to product quantification (Table 2.1). To find the optimum temperature for all three enzyme-ligand combinations, reactions were run between 5 °C and 70 °C. To find the optimum pH, the NaPi buffer was adjusted to values ranging from 5 to 10 (in increments of 0.5). To gain insight into the haemolymph pH of *C. fornicata in situ*, 141 fresh limpets were collected in March 2019. Haemolymph was isolated from every animal (as previously described) and screened using Mquant® Universal pH indicator strips.

2.3.7 Bacterial culture and antibacterial assays

Laboratory strains of Gram-positive (*Bacillus megaterium, B. subtilis, Micrococcus luteus*) and Gram-negative (*Escherichia coli* K12, *Pantoea agglomerans*) bacteria were sourced from Blades Biological Ltd (Kent, UK). Single colonies were picked from nutrient agar (Thermo Scientific) and cultured overnight in liquid medium at 37 °C, except *P. agglomerans*, which was grown at 30 °C. Optical density values were recorded using a V-1200 spectrophotometer. Once bacterial suspensions reached an OD_{600} value of 1,

cells were pelleted via centrifugation at 1000×*g* for 5 min (room temperature), washed twice in NaPi pH 7.4, and diluted in the same buffer to yield 1 × 10^6 colony forming units (CFUs) per mL.

Upon completion of phenoloxidase assays using 5 mM of substrate ($_L$-DOPA, dopamine, hydroquinone), reaction volumes were centrifuged at 4000×*g* for 5 min (room temperature) using Amicon Ultra Filter Units (Millipore) with a 10 kDa molecular weight cut-off to remove any potential laccase or catecholoxidase enzymes. Reaction filtrates (100 µL) were mixed with bacterial suspensions in a 1:1 ratio and incubated at room temperature for 1 h. Following incubation, samples were diluted serially in NaPi pH 7.4 so that ~200 CFUs were spread onto nutrient agar and allowed to grow at 30 °C (*P. agglomerans*) or 37 °C (all other bacteria) for ≤48 h. Control assays in the absence of substrate, and in the presence of an inhibitor (1 mM PTU), were run to attribute antibacterial activity to laccase- and catecholoxidase-derived products only.

2.3.8 Histology of *Crepidula fornicata* soft tissues

Whole tissue histology of *C. fornicata* was used to screen a subset (*n* = 10) for signs of tissue pigmentation, namely melanin. Intact tissues were separated from limpet shells using a blunt-ended probe, submerged in Davidson's seawater fixative (Hopwood, 1996) for 24 h, and washed in dH_2O prior to storage in 70% ethanol. Samples were dehydrated using an ethanol series, 70%, 80% and 90% for 1 h each, followed by 3 × 1 h in 100% ethanol. These samples were washed twice in HistoClear/HistoChoice for 1 h each prior to immersion in paraffin wax: HistoChoice (1:1) for 1 h. Embedded samples were cut into sections 5–7 µm in thickness (using a Leica RM2245 microtome), adhered to glass slides using egg albumin (~1% w/v), and dried for 24 h. Slides were stained using Cole's haematoxylin and eosin. Stained slides were inspected and imaged using an Olympus BX41 digital microscope with a trinocular viewing head.

2.3.9 Data handling

All values reported here represent the mean ± standard error. Enzyme assays were performed in triplicate on three independent occasions. Michaelis-Menten non-linear regression and Lineweaver-Burk plots were used to calculate K_M and V_{max} values. Antibacterial assays were also performed in triplicate on three independent occasions, with data being analysed using 2-way ANOVA and Tukey's multiple comparison (*post-hoc*) tests. Statistical differences were considered significant when $P \leq 0.05$. Data analyses and visualisations were performed in GraphPad PRISM v7. Histology images were adjusted for contrast and colour balance only.

2.4 Results

2.4.1 Characterising phenoloxidase-like activities in the haemolymph of *Crepidula fornicata*

Using a broad series of known phenoloxidase substrates, enzymatic activity was confirmed in the presence of three ortho-diphenols (Figure 2.2), one para-diphenol (Figure 2.3), one non-phenolic para-diamine (Figure 2.3, and two methoxy-containing phenols (Figure 2.4: Laccase activities of *Crepidula fornicata* haemolymph in the presence of phenolic substrates with methoxy groups in vitro. Protein (1 mg mL−1) was incubated in the presence of each substrate for 10 min. Products derived from the enzymatic oxidation of substrate were observed across several wavelengths (listed in Table 2.1). Values represent the mean ± standard error (*n* = 3 biological replicates made-up of 3 technical replicates each). Enzyme-substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-Menten non-linear regression. Each panel also contains the respective double-reciprocal (Lineweaver-Burk) plot.). At concentrations ≤10 mM for caffeic acid, dopamine, and l- DOPA, ≤15 mM for hydroquinone, ≤20 mM for ABTS and p-phenylenediamine, and ≤50 mM for syringaldazine, kinetic data were calculated using the Michaelis-Menten equation and Lineweaver-Burk intercepts (Table 2.2 Kinetic properties of laccase and catecholoxidase activities). Goodness of fit

values (R2) for all regressions ranged from 0.74 to 0.96. The Michaelis constant KM for all three ortho- diphenols was <1.5 mM, with l-DOPA being the lowest at 0.26 mM, which suggests it is the preferred substrate *in vivo*. Hydroquinone (p-diphenol) (Figure 2.2) had a similarly low KM value of 2.05 mM, however, its maximum velocity (Vmax) of ~4.4 U was 3-fold higher than l-DOPA (Figure 2.2) and 1.8-fold higher than dopamine (1.4 U and 2.5 U, respectively;Figure 2.2,Table 2.2 Kinetic properties of laccase and catecholoxidase activities). The highest V_{max} value of 5.7 U was recorded for the exogenous substrate ABTS (a methoxy-containing phenol), but this was accompanied by the highest $K_{M\ value}$ of 21 mM – indicating the enzyme-ligand complex is not stable. Under the described experimental conditions, no measurable activity in the presence of three common monophenols (4-hydroxyanisole, tyramine, $_{L}$-tyrosine) or a single *meta*-diphenol (DHPPA) using concentrations from 0.1 mM to >25 mM was observed. Additionally, the use of sodium dodecyl sulphate (SDS) at concentrations in excess of critical micelle formation (~3.5 mM) did not enhance enzymatic activity of the haemolymph protein.

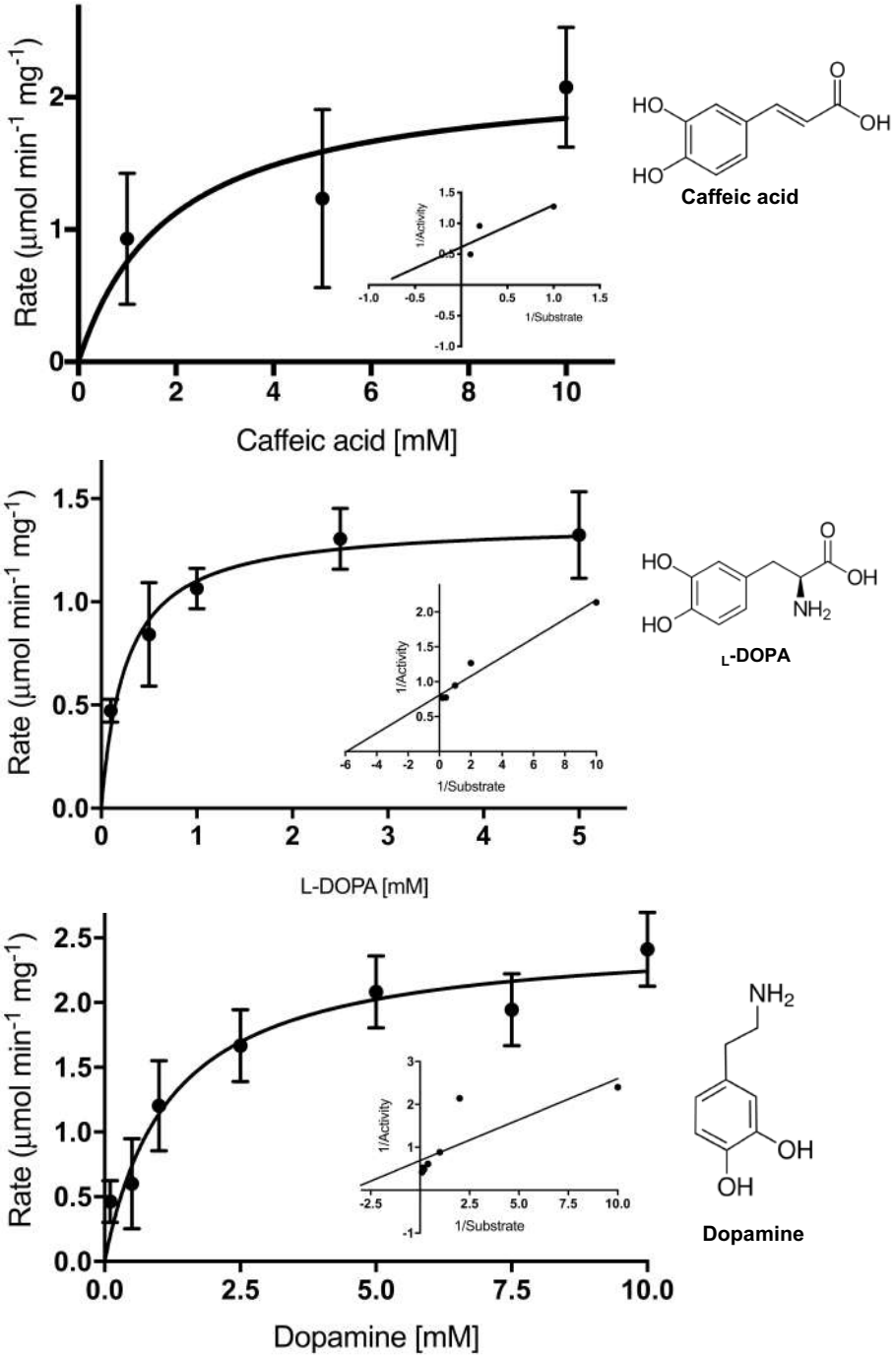

Figure 2.2 Catecholoxidase activities of *Crepidula fornicata* haemolymph protein in the presence of ortho-diphenolic substrates in vitro. Protein (1 mg mL−1) was incubated in the presence of each substrate for 10 min. Products derived from the enzymatic oxidation oof substrate were observed across several wavelengths (listed in Table 2.1). Values represent the mean ± standard error *(n* = 3 biological replicates made-up of 3 technical replicates each). Enzyme-substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-Menten non-linear regression. Each panel also contains the respective double- reciprocal (Lineweaver-Burk) plot. The chemical structure of each substrate is also shown

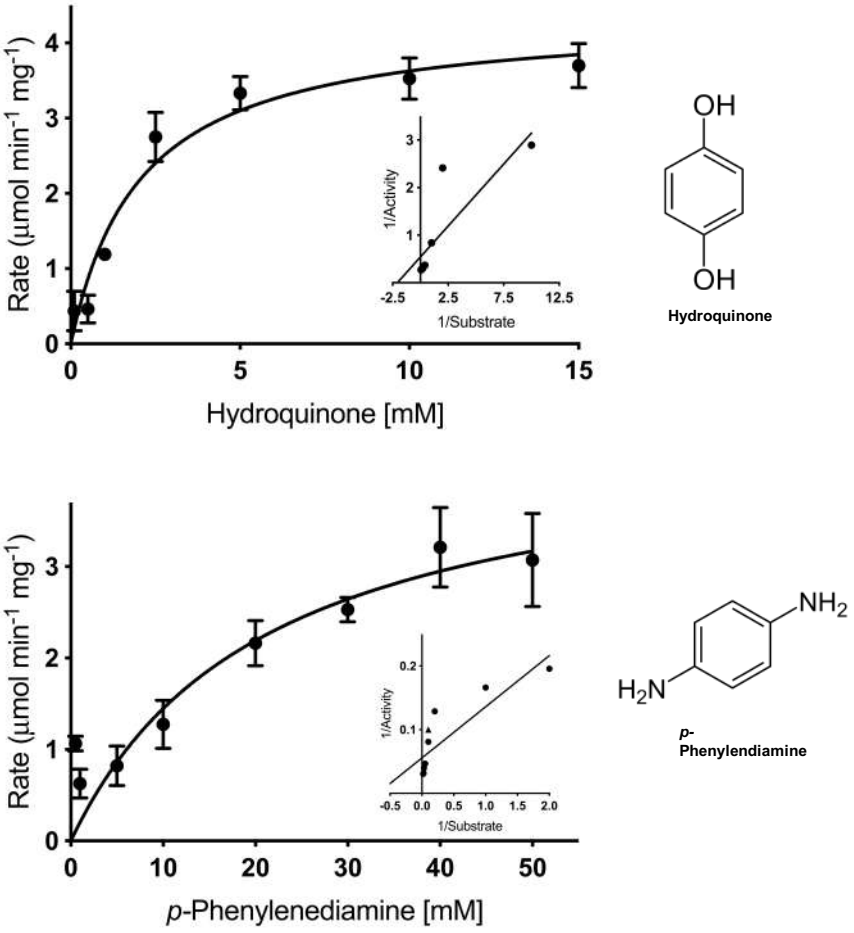

Figure 2.3 Laccase activities of *Crepidula fornicata* haemolymph in the presence of a para-diphenol (hydroquinone), and a non-phenolic (p-phenylenediamine) substrate in vitro. Protein (1 mg mL−1) was incubated in the presence of each substrate for 10 min. Products derived from the enzymatic oxidation of substrate were observed across several wavelengths (listed in Table 2.1). Values represent the mean ± standard error *(n* = 3 biological replicates made-up of 3 technical replicates each). Enzyme- substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-Menten non-linear regression. Each panel also contains the respective double-reciprocal (Lineweaver-Burk) plot. The chemical structure of each substrate is also shown

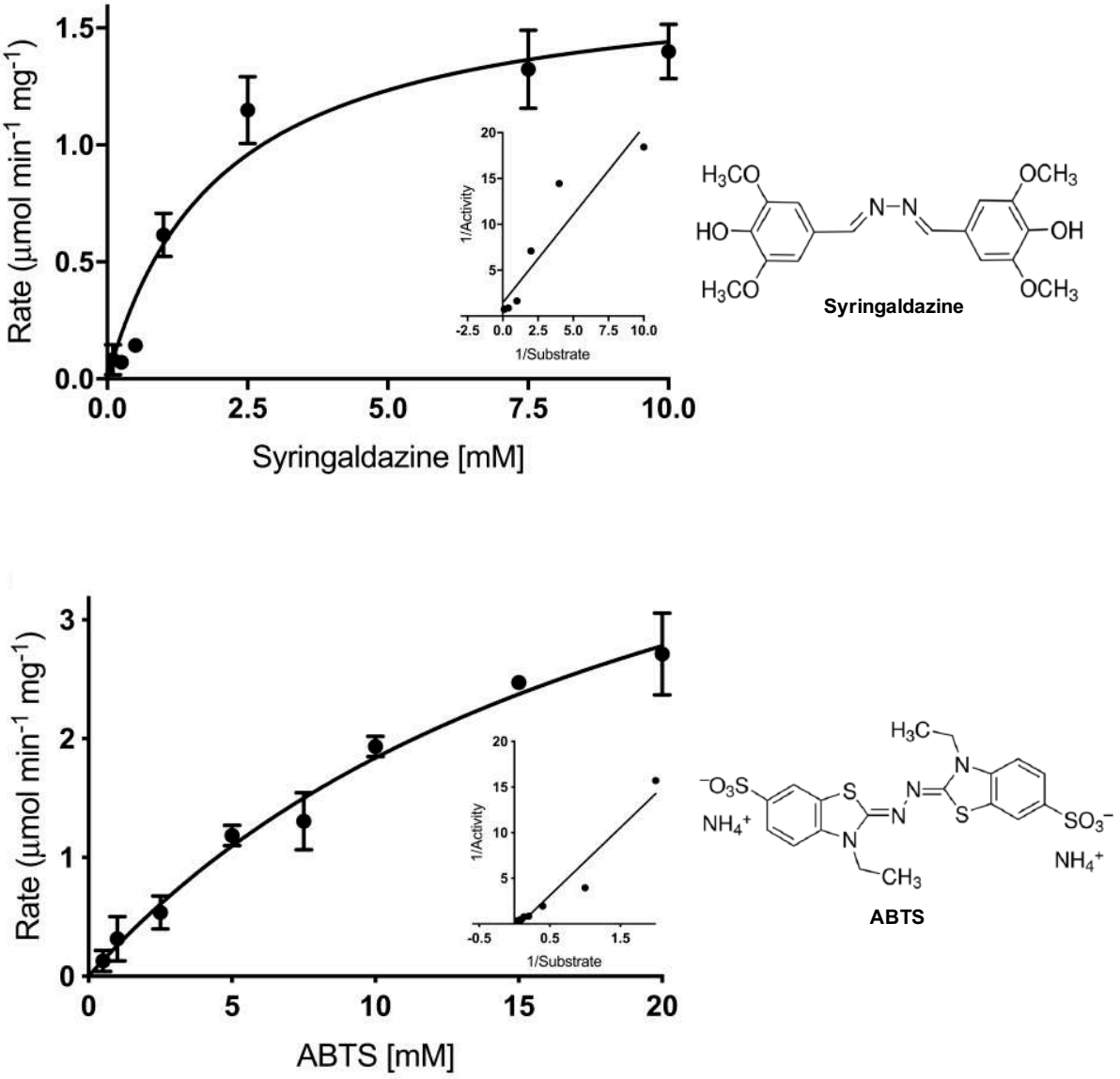

Figure 2.4: Laccase activities of *Crepidula fornicata* haemolymph in the presence of phenolic substrates with methoxy groups in vitro. Protein (1 mg mL−1) was incubated in the presence of each substrate for 10 min. Products derived from the enzymatic oxidation of substrate were observed across several wavelengths (listed in Table 2.1). Values represent the mean ± standard error (*n* = 3 biological replicates made-up of 3 technical replicates each). Enzyme-substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-Menten non-linear regression. Each panel also contains the respective double-reciprocal (Lineweaver-Burk) plot.

+ABTS - 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid

Table 2.2 Kinetic properties of laccase and catecholoxidase activities

Substrate	Substrate class	Enzyme	K_M	V_{max} (μmol min^{-1} mg^{-1})	R^2
ABTS	methoxy-phenol	Laccase	21.1 ± 4.82	5.71 ± 0.81	0.96
Hydroquinone	*para*-diphenol	Laccase	2.05 ± 0.38	4.37 ± 0.26	0.79
p-Phenylenediamine	non-phenolic	Laccase	21.2 ± 8.3	4.51 ± 0.74	0.82
Syringaldazine	methoxy-phenol	Laccase	2.01 ± 0.44	1.73 ± 0.13	0.93
Caffeic acid	*ortho*-diphenol	Non-specific	1.11 ± 0.43	1.63 ± 0.62	0.74
DHPPA	*meta*-diphenol	Non-specific	-	-	-
L-DOPA	*ortho*-diphenol	Non-specific	0.26 ± 0.07	1.4 ± 0.08	0.82
Dopamine	*ortho*-diphenol	Non-specific	1.21 ± 0.32	2.51 ± 0.18	0.85
4-Hydroxyanisole	mono-phenol	Tyrosinase	-	-	-
Tyramine	mono-phenol	Tyrosinase	-	-	-
L-Tyrosine	mono-phenol	Tyrosinase	-	-	-

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DHPPA, 3,5-dihydroxyphenylpropionoic acid
L-DOPA, L-3,4-dihydroxyphenylalanine
Syringaldazine, 4-hydroxy-3,5-dimethoxybenzaldehyde azine

Enzyme-catalysed turnover of substrates was assessed further using a series of known phenoloxidase inhibitors (Table 2.3). Citric acid and benzoic acid are non-specific inhibitors of PO activity, and concentrations in excess of 0.1 mM thwarted product formation by 71–100%, regardless of the substrate used. As the active sites of POs use copper to facilitate catalysis, the metal chelator EDTA decreased dopamine oxidation by 86–100% and hydroquinone oxidation by 72–100% (Table 2.3). Using the laccase-specific inhibitor CTAB, and the laccase-specific substrate hydroquinone, activity diminished by 100%. Using CTAB at the highest concentration of 1 mM in the presence of dopamine, however, did not eliminate all enzyme activity (~10% left) – indicating the presence of a second phenoloxidase. Using the tyrosinase- and catecholoxidase-specific inhibitor 4-hexylresorcinol (at 0.5 and 1 mM), enzyme activity decreased by

>80%. 4-hexylresorcinol had little impact when hydroquinone replaced dopamine, with 90% of enzyme activity remaining intact (Table 2.3).

To gain insight into endogenous conditions, fresh limpets were collected, haemolymph was isolated, and the pH measured. Values ranged from 7 to 9 with an average pH of 7.5 ± 0.15 (n = 141). Following this, representatives of the three substrate classes with the highest V_{max} values were selected, ABTS (5.7 U), dopamine (2.5 U) and hydroquinone (4.4 U; Table 2.2) and determined activity across a pH (5- 10) gradient *in vitro* (Figure 2.5A). Maximum levels of ABTS oxidation occurred at pH 5.5, whereas the enzymatic turnover of dopamine and hydroquinone (into dopaminechrome and benzoquinone) were highest at pH 7 and 8, respectively. Subjecting the haemolymph samples to increasing temperatures from 5 to 70 °C, revealed temperature optima of 35 °C for dopamine and 45 °C for hydroquinone (Figure 2.5B). Under these conditions, there were no substantial differences in product formation from hydroquinone between temperatures 35 and 50 °C (89–100% inclusive).

Table 2.3 Inhibition of laccase and catecholoxidase activities

Inhibitors	Enzyme target	Inhibitor conc.	Inhibition (%)	
			Dopamine#	Hydroquinone#
Benzoic acid	Non-specific	1 mM	95.9	76.9
		5 mM	98.7	84.1
		10 mM	100	100
Citric acid	Non-specific	1 mM	87.4	71.3
		5 mM	88.9	78.9
		10 mM	100	100
CTAB	Laccase	1 mM	67	98
		5 mM	84	100
		10 mM	90.7	100
EDTA	Non-specific	1 mM	85.6	72.3
		5 mM	92.6	97.6
		10 mM	100	100
4-hexylresorcinol	CO, TY	1 mM	57	-
		5 mM	84	-
		10 mM	82.8	10.3
PTU	Non-specific	1 mM	84.1	93.5
		5 mM	89.7	98.7
		10 mM	90	100

#, substrates were used at a standard concentration of 5 mM for all inhibition assays CTAB, Cetrimonium bromide;
CO, catecholoxidase
EDTA, ethylenediaminetetraacetic acid
PTU, phenylthiourea
TY, tyrosinase;

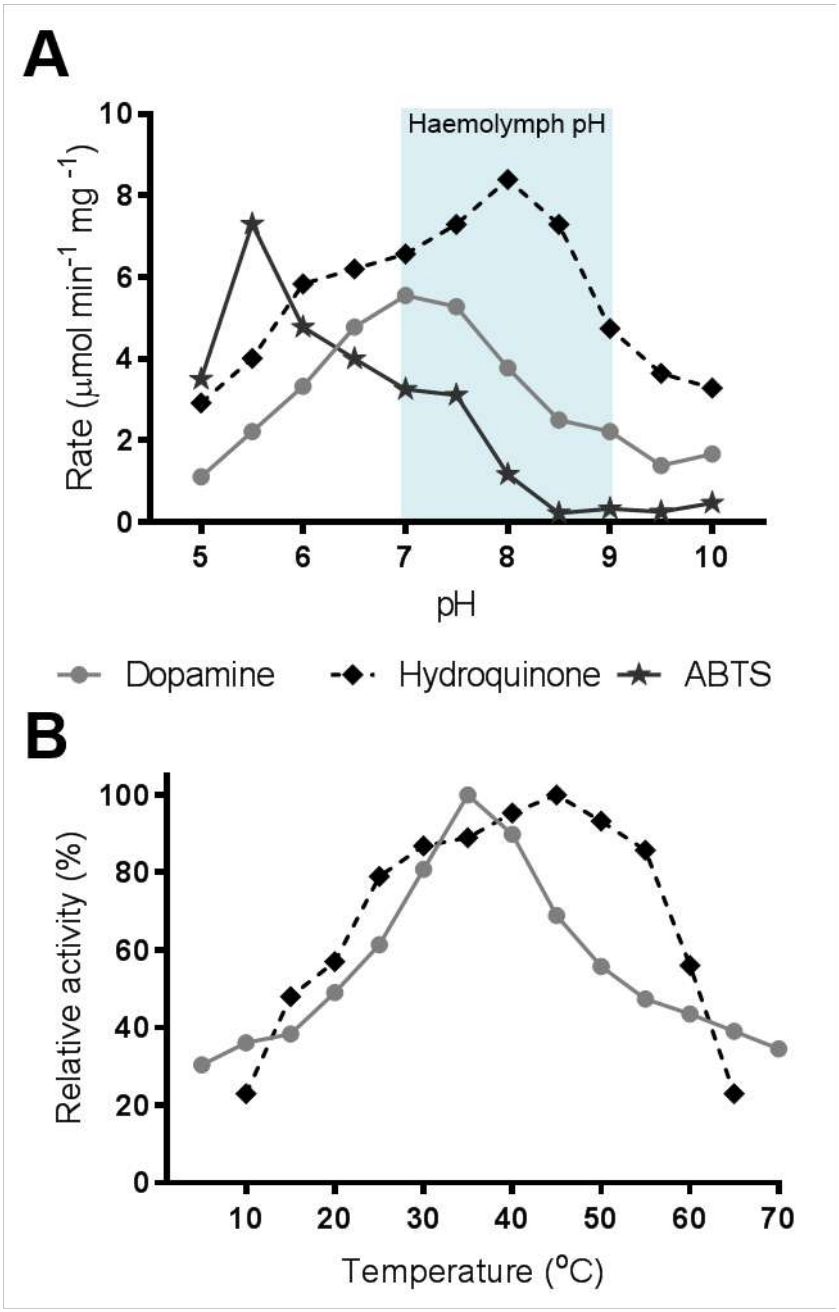

Figure 2.5: Effect of pH and temperature on laccase and catecholoxidase activities in the haemolymph of *Crepidula fornicata*. Protein (1 mg mL-1) was incubated in the presence of either substrate for 10 mins across the pH range 5-10 (A) and the temperature range 5-70 °C (B). Activity (rate) was measured as the amount of product formed from the oxidation of dopamine (into dopachrome), hydroquinone (into benzoquinone), and ABTS (into $ABTS^{+}$). In (A), the pH range of fresh (*ex vivo*) limpet haemolymph (n= 141) is shaded blue. In (B), values are expressed as percentage of the mean maximum value for dopamine (35 °C) and hydroquinone (45 °C).

2.4.2 Antibacterial potency of enzymatic reaction products

Using both *ortho* and *para* isomers of diphenols (dopamine, L-DOPA and hydroquinone) at a standardised concentration of 5 mM, the antibacterial properties of their respective oxidised quinone (by)products (dopaminechrome, DOPAchrome, benzoquinone) was tested. Overall, the exposure of bacteria to these enzyme-derived products led to significant reductions in CFUs; $F(3, 40) = 254.7$, $P < 0.0001$ (Figure 2.6). The majority of variation within the data, 87%, can be attributed to the type of substrate used. Gram-negative bacteria were sensitive to all reaction products, in particular, oxidised hydroquinone (i.e., benzoquinone) was highly effective against *P. agglomerans* – reducing CFUs by 95%. Conversely, Gram-positive bacteria were less sensitive to reaction products, e.g., oxidised L-DOPA (i.e., DOPAchrome) led to the smallest decline of 24% when exposed to *B. subtilis*. With that said, microbial target was determined to be a significant factor ($F(4, 40) = 7.03$, $P = 0.002$) and accounts for 3.2% of the variation within the data.

The bactericidal potency of diphenols can be ranked hydroquinone > dopamine > L-DOPA, and after 1-h incubation each one caused sufficient damage to prevent replication, immobilise and/or kill the microbes. Although the use of L-DOPA did lead to decreases in *B. megaterium* and *B subtilis* CFUs, neither were significantly different to the respective controls ($P = 0.099$ and $P = 0.335$).

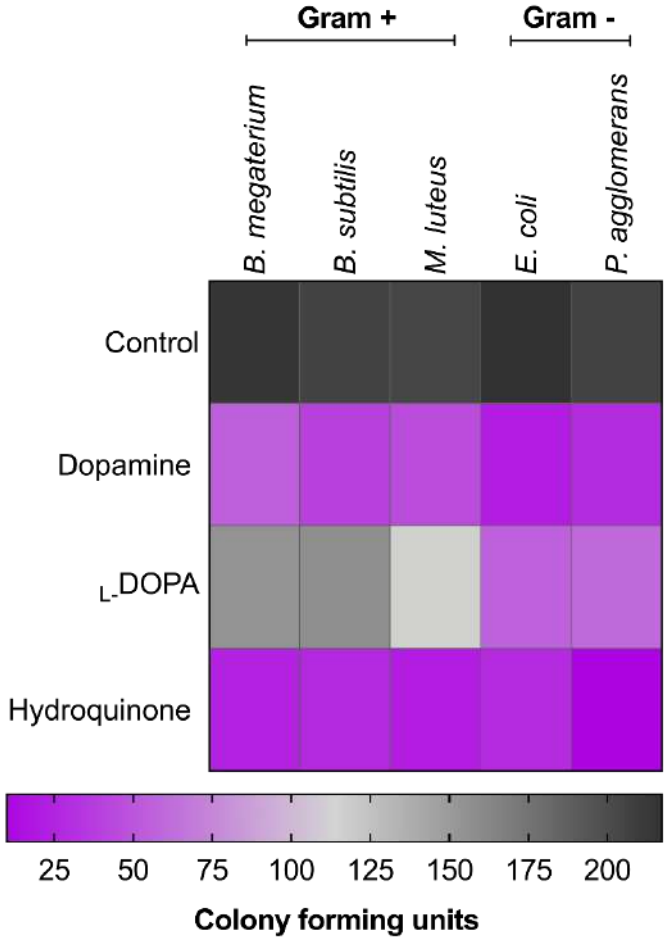

Figure 2.6: Antibacterial effects of laccase- and catecholoxidase-derived reaction products in vitro. Cell-free haemolymph protein (1 mg mL−1) from *Crepidula fornicata* was incubated with ortho-diphenolic (dopamine, l-DOPA) and para-diphenolic (hydroquinone) substrate substrates from 10 min. Post-incubation, proteins were filtered (>10 kDa cut-off) using centrifugation, and the subsequent reaction mixtures containing the oxidised products were incubated with Gram-positive (*Bacillus megaterium, Bacillus subtilis, Micrococcus luteus*) and Gram-negative (*Escherichia coli,, Pantoea agglomerans*) bacteria. The heat map depicts the mean number of colony forming units for treated microbes (n=3) and controls (substrates were omitted).

2.4.3 Histological observations of *Crepidula fornicata* tissues

Using wax (H & E) histology, discrete brown/black pigmentation (eumelanin) was observed in the lining of the gill tissue, barrier epithelium, connective tissue, and border cells of the foot musculature (Figure 2.7). These melanic-deposits accumulated at the apical surface of epithelial cells (Figure 2.7D) but did not appear pathologic (no signs of infection or trauma). The cellular arrangement is uniform and there is no clear sign of a host response, e.g., haemocyte infiltration or encapsulation, to accompany the melanisation (which can be found in compromised tissues of invertebrates). Interestingly, yellowish pigmentation reminiscent of the lysosomal degradation product, lipofuscin, was visible in the digestive gland intra- and inter-tubular structures Figure 2.7B), as well as connective tissue (Figure 2.7B and F).

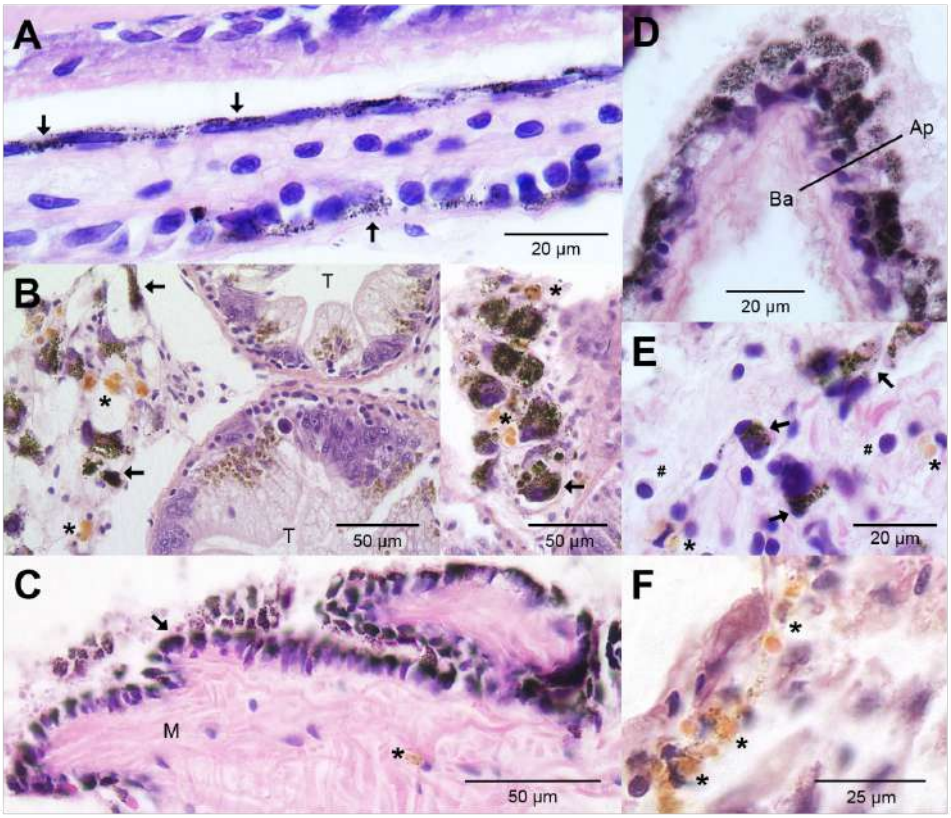

Figure 2.7: Tissue histology of *Crepidula fornicata*. Photomicrographs depict transverse sections of gill tissue (A), the digestive gland (B), the foot musculature (C), barrier epithelium (D), and connective tissues (E and F). In all images, arrows point to melanin deposits within a variety of cell types, and each asterisk (*) indicates lipofuscin-like material. Ap, apical; Ba, basal; M, muscle; T, tubule. In (E), a hashtag (#) denotes the presence of a haemocyte.

2.5 Discussion

In this chapter, strong evidence that proteins present in the acellular haemolymph of *Crepidula. fornicata* display phenoloxidase-like activities are presented. The haemolymph tested negative for tyrosinase (monophenolase) activity and also appeared incapable of oxidising the *meta*-diphenol DHPPA. The low Michaelis' constant (K_M) values for both laccase-type (*para*) and catecholoxidase-type (*ortho*) substrates suggested the enzyme-ligand interactions were stable (Table 2.2), except for the methoxy-containing phenols (syringaldazine and ABTS) with calculated values in excess of 20 mM. The oxidation of general *o*-diphenols (e.g., dopamine) and the more-specific *p*-diphenol (hydroquinone) were inhibited by the metal chelator EDTA, and in doing so, confirmed the activities to be derived from metalloenzymes – as seen in *C. gigas* (see Luna-Acosta *et al*. 2010). In the presence of hydroquinone, the laccase specific inhibitor CTAB prevented all product formation. However, in the presence of dopamine, CTAB inhibited activity by a maximum of 91%. Moreover, the highest concentration of the tyrosinase/catecholoxidase-specific inhibitor 4-hexylresorcinol (1 mM) hindered activity by ~80% and ~10% in the presence of dopamine and hydroquinone, respectively (Table 2.3). These data endorse the presence of two independent diphenoloxidases within *C. fornicata* haemolymph, namely laccase and catecholoxidase. Regarding the aromatic amine, *p*-phenylenediamine, I obtained a $K_{M\ value}$ of 2.01 mM, which is in line with those published for *Biomphalaria* sp., 1.19– 1.45 mM (Le Clec'h *et al*., 2016). Using similar assay conditions, Le Bris *et al*. (2014) reported a much higher K_M value of 13.5 mM for *Haliotis tuberculata* (Linnaeus, 1758), and Luna-Acosta *et al*. (2011) recorded the highest at 45 mM when studying *C. gigas*. A pH optima of 7 -8 was observed, and a temperature optima at 35 °C and 45 °C *in vitro* for dopamine and hydroquinone, respectively (Figure 2.5). The *ex vivo* pH of *C. fornicata* haemolymph varied between 7 and 9, which suggests it is more suited for hydroquinone oxidation (Figure 2.5). These data fall within the reported ranges for

both laccase-type and catecholoxidase-type enzymes. For example, when using *p*-phenylenediamine, the pH maximum was 8.5 for *Biomphalaria* sp. (see Le Clec'h *et al*. 2016), 8.2 for *H. tuberculata* (see Le Bris *et al*. 2014), and 8.4 for *Venerupis philippinarum* (Adams & Reeve, 1850; see Le Bris *et al*. 2013). When using an *o*-diphenol, optimal activity was achieved at pH 8 for *Saccostrea glomerata* (Gould, 1850; see Aladaileh *et al*. 2007), pH 6–7.5 for *C. virginica* (see Jordan and Deaton 2005) and *Chlamys farreri* (see Hushan and Guangyou 1999).

Previously, Pires *et al*. (2000) detected three catecholamines – dopamine, L-DOPA and norepinephrine – in *C. fornicata* larvae and juveniles (using high performance liquid chromatography). Inhibition of tyrosine hydroxylase and dopamine-β-hydroxylase using α-methyl-DL-m-tyrosine and diethyldithiocarbamate reduced levels of catecholamines by 20–50% and interfered with morphogenesis. Low K_M values < 1.5 mM for two of the catecholamines mentioned above were calculated (Figure 2.2,Table 2.2). The data suggests that L-DOPA and dopamine are endogenous substrates of phenoloxidase(s) in *C. fornicata* adults.

Whilst bioprospecting molluscs for antiseptic compounds, Defer *et al*. (2009) prepared some acidic extracts of *C. fornicata* tissues and recorded antibacterial activity against *M. luteus* (Gram-positive) and *Listonella anguillarum* (Bergeman 1909; Gram-negative), and virustatic properties toward *Herpes simplex virus* type 1 (viral replication was reduced by 40% when compared to the control). This chapter also describes anti-infective properties of *C. fornicata* haemolymph (Figure 2.6), yet importantly, the evidence implies the mechanism of action is of enzymatic origin. The following points contend that CFU declines were due to a combination of the noxious intermediates of laccase and/or catecholoxidase reaction products: (1) in the absence of any substrate and in the presence of the phenoloxidase inhibitor PTU, CFU numbers were in line with controls (>97%); (2) in the absence of haemolymph protein, no measurable antibacterial activity was observed; (3) using a 10 kDa filter to remove potential

phenoloxidase(s) from the reaction mixtures prior to microbial exposure reduced the likelihood of proteinaceous macromolecules interacting directly with the targets. The penultimate step of the eumelanin synthesis pathway is 5,6-dihydroxyindole (DHI) formation, which can happen spontaneously or enzymatically from DOPA- derivatives and is known to have direct antimicrobial activity (J. Zhao *et al*., 2007). DOPAchromes themselves are unstable, as are the cytotoxic oxidising and nitrosative radicals produced during phenol oxidation (Coates and Nairn, 2014).

Traditionally, laccases have not been considered part of the invertebrate innate immune system, despite their capacity to metabolise melanin precursors, i.e., phenols. First, Luna-Acosta *et al*. (2011) noted restricted growth (>30%) of the marine pathogens *Vibrio splendidus* LGP32 and *Vibrio aestuarianus* 02/41 after treatment with *C. gigas* haemocyte lysate supernatant and two substrates, *p*-phenylenediamine and L-DOPA. The anti-Vibrio properties were thwarted by the addition of the phenoloxidase inhibitor, PTU. The data collected in this chapter complement these earlier observations. The reaction products derived from hydroquinone and dopamine oxidation were highly effective against all microbes tested (Figure 2.6) but were indistinguishable from controls when PTU was added. In contrast, L-DOPA oxidised (by)products were not as effective against Gram-positive bacteria, notably *Bacillus* sp. Similar measurements were taken with regards the relatively weak antimicrobial activity of crayfish (*Pacifastacus leniusculus;* Dana, 1852) phenoloxidase and horseshoe crab (*Limulus polyphemus*; Linnaeus, 1758)haemocyanin-derived phenoloxidase when L-DOPA was used compared to other diphenols (e.g., 4-*tert*-butylcatechol) at the same concentration (Cerenius *et al*., 2010b; Coates and Talbot, 2018). Recently, Shi *et al*. (2017) challenged Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) with *Vibrio parahaemolyticus*, *Micrococcus lysodeikticus* and white spot syndrome virus (WSSV) and noted increased expression of laccase-specific mRNAs. In a separate experiment, the authors silenced the laccase gene using dsRNA, which increased shrimp susceptibility to both bacterial types, and caused >20% higher

mortality. In a subsequent study, Chen *et al*. (2020) identified a second laccase gene (*LvLac*2) from *Penaeus vannamei* (Boone, 1931) within the epidermal layers of the carapace that was also linked to immune activity. Injection of shrimp with WSSV or *V. alginolyticus* led to increased expression of the *LvLac*2 gene, and the oxidative stress-associated transcription factor NF-E2. Additionally, injection of dsRNA for *LvLac*2 reduced the survivorship of shrimp when challenged with WSSV. A notable side-effect of eliminating laccase gene expression was an increase in tissue damage found in the hepatopancreas of shrimp immune- stimulated with β-glucans. The authors concluded that it was caused by oxidative damage in the absence of laccase, and that laccase likely has multiple functions.

Phenoloxidases are distributed widely amongst metazoans, microbes, and plants. Their roles differ depending on the organism, for example: plant polyphenoloxidases and arthropod tyrosinases are involved in host counter-responses to disease-causing agents, while fungal laccases act as enzymatic antioxidants/detoxicants and assist in lignocellulose degradation (Baldrian, 2006; Cerenius *et al*., 2008; Janusz *et al*., 2020). The histological screen of *C. fornicata* of solid tissues revealed the presence of melanin and lipofuscin-like pigments across diverse tissue types. In previous work by Tiley *et al*. (2018, 2019), brown inclusion bodies – bulbous or conical in shape – were characterised in the digestive gland of another gastropod, the queen conch *Lobatus gigas* (Linnaeus, 1758). Using a combination of techniques, including histochemical staining and electron microscopy, these were confirmed to be aggregates of melanin, iron, glycoproteins and mucopolysaccharides. In line with the observations made of slipper limpet tissues herein, Tiley *et al*. (2018, 2019) did not find any evidence of damage, inflammation or infection (e.g., apicomplexan parasites), however, they did observe such pigmented deposits in several other areas, including ganglia. These studies may go some way to explain the presence of lipofuscin – a lysosomal degradation product in the digestive gland and connective tissues of *C. fornicata* (Figure 2.7). Lipochrome in the form of small yellow aggregates can be considered stage 1 lipofuscin, which can go on to form immature (stage 2) brown bodies. These brown bodies are often associated

with pathogen clearance, mineral storage and cellular senescence, and the darker pigmentation can be attributed to melanin accumulation from oxidation reactions (Valembois *et al*., 1994).

The published genomes of several bivalves, *C. gigas* (see Zhang *et al*. 2012), *C. farreri* (Johnes & Preston, 1904; see Li *et al*. 2017) and *Pinctada fucata martensii* (Gould, 1850; see Du *et al*. 2017), revealed major gene expansion (sub/neo-functionalisation) events for phenoloxidases, notably tyrosinases and laccases. Moreover, expression of laccase and tyrosinase-like protein mRNAs were up-regulated in regions such as the mantle and digestive gland, which further implies multiple roles in development, detoxification, and defence. Interestingly, the expression of at least two laccase genes has been recorded in the epithelium, muscle, intestine, stomach, hepatopancreas, gill, haemocytes, nerve tissue and heart of penaeid shrimp (Shi *et al*., 2017; Chen *et al*., 2020).

2.6 Conclusion

This chapter establishes that enzymes present in the haemolymph of the invasive gastropod *C. fornicata can* accept diphenolic substrates and convert them into quinones (melanin precursors) in a manner similar to laccases (EC 1.10.3.2) and/or catecholoxidases (EC 1.10.3.1). The resulting (by)products are cytotoxic and possess broad-spectrum antibacterial properties. The capacity of this gastropod to generate melanin is evidenced further by the distribution of this pigment across many tissues. Taken together, evidence suggests that two constitutive phenoloxidases contribute to biological defences in *C. fornicata.*

Chapter 3 Interrogating the potential role of *Crepidula fornicata* as a sink for Vibrio-like bacteria

3.1 Abstract

Surprisingly, there is a large gap in knowledge regarding the disease status of the invasive, alien, slipper limpet *Crepidula fornicata*. To help address this, a yearlong disease survey was carried out across two sites in south Wales UK – subtidal Swansea Bay (1) and intertidal Milford Haven (2). In total, 1,800 limpets were collected between January 2019 and December 2019, and screened systematically for haemolymph bacterial burdens (colony forming units) using both general and vibrio-selective growth media (TSA +2% NaCl and TCBS, respectively), a PCR-based assay targeting *Vibrio* spp., and whole tissue histology. Overall, >99% of haemolymph samples contained cultivable bacterial colony forming units, and 83% of limpets tested positive for the presence of Vibrio-like bacteria through end-point PCR (amplicons were identified *via* Sanger sequencing). Less than 1% of limpet haemolymph samples plated on the Vibrio-selective TCBS medium showed no measurable signs of growth. *Vibrio* presence did not vary greatly across both sites; however a strong temporal effect was observed across seasons – the summer months showing significantly higher bacterial loads. Using binomial logistic regression models, larger (older) individuals were more likely to harbour Vibrios. The model also demonstrated that growth of bacterial colony forming units on TCBS medium, was a key predictor for an individual testing positive via PCR-based detection. Importantly, histological assessment of >350 limpets revealed little evidence of inflammation, sepsis or immune-reactivity despite the gross bacterial presence. Therefore, these data suggest that *C. fornicata* are not highly susceptible to bacteriosis at the two sites surveyed.

3.2 Introduction

Molluscs form an important economic and ecological component of marine ecosystems (FAO, 2017; Sowa *et al*., 2019). Along with their economical role through aquaculture and wild-harvest industries, they aid in determining community structure, nutrient cycling, act as food sources for higher trophic levels, stabilize shorelines, and maintain water quality (Goss-Custard *et al*., 2004; Jansen *et al*., 2012; Kellogg *et al*., 2014; Walles *et al*., 2015; Tomatsuri and Kon, 2017). As some molluscs filter-feed, this can lead to the accumulation of diverse bacteria (Romalde *et al*., 2014). *Crepidula fornicata* is an invasive alien limpet, first introduced to Europe in the 1870s with the American oyster *Crassostrea virginica,* and subsequently spread along the European coast (Blanchard, 1997). *Crepidula fornicata* became established in Welsh waters in the 1950s (Cole and Baird, 1953), and has gained notoriety as an "oyster pest" due to its supposed effect of trophic competition with *C. gigas* and *Ostrea edulis.* Though some studies have presented evidence to the contrary, for example, de Montaudouin *et al*. (1999) was able to demonstrate in laboratory trials that *C. gigas* growth, and macrozoobenthic density and diversity was not negatively impacted by the presence of *C. fornicata*. Through the use of natural history records, Hayer *et al*. (2019) reconstructed the changes in distribution and diversity over the last 200 years in the North Sea, and concluded that the decline of *O. edulis* was near completion before the introduction of *C. fornicata* . Conversely, Le Pape *et al*. (2004) showed that the presence of *C. fornicata* in flat-fish nurseries can have a negative impact, reducing the density of young-of-the-year sole by limiting their ability to bury into the sediment. Despite an exhaustive literature search, there appears to be little focus placed on investigating the disease status of this invasive species, or alternatively, this species is resistant to common disease targets. Le Cam and Viard (2011) investigated the temporal prevalence of a boring sponge, *Cliona celata,* in a French population of *C. fornicata.* They examined a total of 12,049 individuals over the course of 37 months and found that on average 43% of the individuals were infected by the sponge. *Crepidula fornicata* individuals infested with *C. celata* generated extra shell material as a result.

The disease status of a species is an important factor to consider when deciding if its presence can be detrimental to the health status of other co-located animals, e.g. commercially sensitive oysters (*C. gigas*) and mussels *(Mytilus edulis*).

Bacteria have diverse and important roles in marine environments, they perform functions such as acting as drivers for biogeochemical cycles (Hawley *et al*., 2017), and supply higher tropic levels with materials and energy sources (De Carvalho and Caramujo, 2012). Though many bacteria play critical roles in marine ecology, pathogenic bacteria can have extreme negative effects on a wide range of species, notably bivalves (reviewed by Travers *et al*. 2014). One particular bacterial genus that blights marine invertebrates is *Vibrio*, which contains highly virulent strains associated with mass mortalities of commercially important bivalves (e.g., *Vibrio aestuarianus* in Pacific oysters; Labreuche *et al.* 2006; Lupo *et al.* 2019), and crustaceans (e.g., *Vibrio parahaemolyticus* causes acute hepatopancreatic necrosis disease in penaeid shrimp; Kumar *et al*. 2020). Early studies of *Vibrio* species dates back to 1854, when Florence, Italy was in the midst of a cholera outbreak. Filippo Pacini was an anatomist who was able to identify millions of what he describes as "vibrions" in the faeces and intestinal mucosa of patients who died as a result. Pacini isolated the agent that he thought was responsible for the cholera outbreak by processing the specimens with water, salt, and sublimate. Around the same time, John Snow (1813-1858) was studying the epidemiology of cholera in a number of English cities including Birmingham, London, and Manchester. Robert Koch (1843-1910) was able to obtain pure cultures of *V. cholerae* on gelatine plates almost 30 years later. The first non- pathogenic *Vibrio* were isolated from the aquatic environment in the late 1880s by the Dutch microbiologist Martinus Beijerinck (1851-1931).

Vibrio are Gram-negative, curved rods of the class *gamma-Proteobacteria* (Williams *et al*., 2010). The genus *Vibrio* comprises over 100 species grouped into 14 clades (Romalde *et al*., 2014).*Vibrio* are almost ubiquitous in aquatic environments, found naturally in

marine, estuarine, and freshwater. They can survive in a diverse range of habitats, having the ability to colonize fish and other marine invertebrates, associated with plankton and algae, and having the capacity to form biofilms on biotic and abiotic surfaces (Reen *et al*., 2006).

Many *Vibrio* species are harmless, yet some can cause disease in both humans and invertebrates (Froelich and Noble, 2016) with ~12 *Vibrio* spp. that cause illness in humans –usually through the ingestion of contaminated food. The major agents of shellfish poisoning are *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae,* leading to gastroenteritis, wound infections, and septicaemia. *Vibrio parahaemolyticus* is responsible for the highest incidences of seafood-associated bacterial gastroenteritis in a number of countries, e.g. United States of America and Asia (Liu *et al*., 2004; Scallan *et al*., 2011). There have been repeated episodes of mass mortality of cultured bivalve molluscs with *Vibrio* being the main aetiological agent of disease (Travers *et al*., 2014; Rojas *et al*., 2015; Oden *et al*., 2016; Wei *et al*., 2019).

Historically, cases of *Vibrio* infections have a strong seasonal occurrence, with most outbreaks taking place during the summer and early autumn, corresponding to increased temperatures (Cabanillas-Beltrán *et al*., 2006). Seawater temperature is a major environmental driver of disease and can influence host susceptibility to infection by modulating aspects of the innate immune defences (Coates and Söderhäll, 2020). Higher transmission and proliferation of *Vibrio* spp. are dependent on upregulation of key virulence factors associated with motility, host degradation, secretion, and antimicrobial resistance. An example of this can be seen in the bleaching of the coral *Pocillopora damicornis* in the Red Sea. The aetiological agent is *Vibrio shiloi*, with an increase in temperature leading to the initiation of a number of virulence mechanisms by the pathogen (Banin *et al*., 2003); production of an adhesin that enables it to stick to a beta-galactoside-containing receptor in the coral mucus, penetrate into the coral epidermis, multiply intracellularly, differentiate into a viable- but-not-culturable state and produce toxins that inhibit photosynthesis and lyse the symbiotic zooanthellae

(Rosenberg and Ben-Haim, 2002). Recently, Wagley *et al*. (2021) characterised this so-called viable but non-culturable (VBNC) state of *V. parahaemolyticus* where the bacterium remains metabolically active, but resistant to cultivation when conditions are unfavourable – demonstrating enhanced capacity to revive and cause disease when conditions become favourable.

Various diagnostic techniques can be used to detect *Vibrio.* One standard method is through microbiological enrichment, namely thiosulfate-citrate-bile salts agar (TCBS), used routinely as the selective medium for *Vibrio*. *Staphylococcus, Flavobacterium, Pseudoalteromonas, Aeromonas* and *Shewanella* isolates can also grow on TCBS, as such, the medium is not entirely restricted to *Vibrio*. *Vibrio* strains that can utilise sucrose form yellow colonies on the medium, whereas the others are greenish (Thompson *et al*., 2004). Molecular methods such as PCR are also used to detect the presence of *Vibrio* (see Vezzulli *et al*. 2012) – a multi-resource approach e.g. a combined usage of PCR, and agar plates can provide the best likelihood of picking up the presence of *Vibrio.*

The aim of this chapter was to perform the first large-scale temporal survey for Vibrio-like bacteria in the alien invader, *C. fornicata,* across two affected locations in Wales, U.K., i.e., Swansea Bay (subtidal sampling within a native oyster restoration zone), and Milford Haven (intertidal sampling among an area of key fisheries/culture industries).

3.3 Material and Methods

3.3.1 Study sites

To detect and monitor the presence of putative vibrios in limpets, 75 samples per site were collected monthly from January 2019-December 2019 and processed using a multi-resource screen. Sampling efforts for both intertidal and subtidal collection of live adult *C. fornicata (*n= 1,800) took place at two sites in the south Wales that are known to have well established populations (Figure 3.1*;* dead limpets were not included in the study). Swansea Bay (n = 900) was sampled subtidally (51.570345, -

3.974591) and Milford Haven (n = 900) was sampled intertidally (51.7042, -4.971295). Swansea Bay is a shallow embayment with depth generally <20 m Ordnance Datum (OD), located on the northern coastline of the Bristol Channel (Kye and Blott, 2014). The bay stretches ~12 km from Mumbles Head to Port Talbot, with the eastern side directly facing the Atlantic Ocean. Swansea Bay's tidal range is one of the largest in North-West Europe with a mean spring tide of 8.6 m and the mean neap tide of 4.1 m (Collins *et al*., 1979; Shackley and Collins, 1984). Sediments are mostly fine and medium sand in the inner bay with an increasing proportion of mud close inshore to the west, due to protection from wave exposure by Mumbles Head, and shallower waters slowing tidal currents (Smith and Shackley, 2006). Historically, Swansea Bay has industrial links to the coal, steel, copper, and fishing industries and presently the main industrial activity is centred at Port Talbot, which is one of the largest integrated steelworks in the UK (Dall'Osto *et al*., 2008). Swansea Bay is a site of recreational usage with designated bathing beaches (Thomas *et al*., 2015) and for several years has been the focus of native oyster restoration.

The Milford Haven Waterway (MHW) is a ria-estuary, an uncommon estuary type restricted in the UK to SW England, and Wales. The MHW is the only example of its kind in Wales and the largest ria-estuary complex in the UK (~ 170 km long). Of the 55 km^2 area covered, over 30% is composed of intertidal habitat (Burton, 2008). Pembroke Marine Special Area of Conservation (SAC) is the UKs third largest SAC. The SAC stretches from north of Abereiddy on the north Pembrokeshire coast to east of Manorbier in the south, and includes the islands of Ramsey, Skomer, Grassholm, Skokholm, the Bishops and Clerks, and The Smalls. The SAC also includes the Milford Haven Waterway. The native oyster *Ostrea edulis*, is part of the estuary feature for the SAC, and is a priority Biodiversity Action Plan species at a UK level. Milford Haven is host to one of only three surviving oyster beds in the U.K (Burton, 2008).

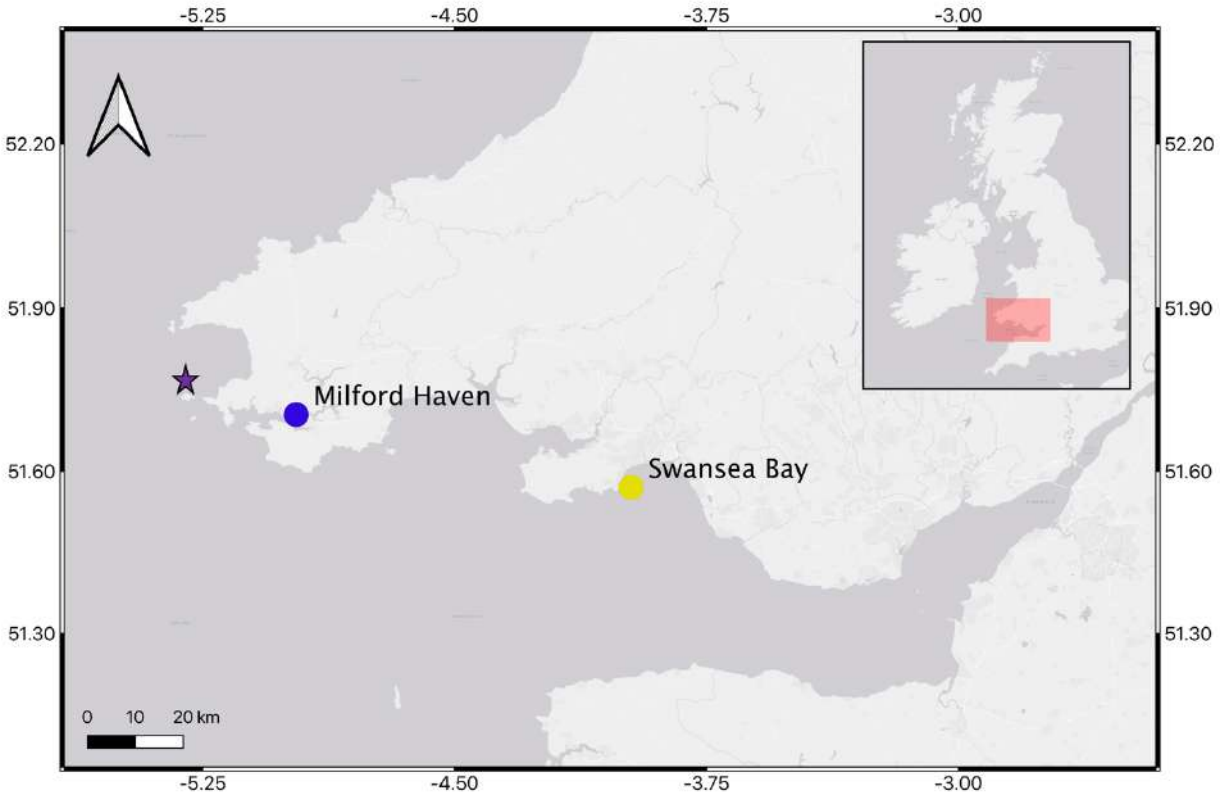

Figure 3.1: Collection sites for slipper limpets (*Crepidula fornicata*) used during this study, Swansea Bay, and Milford Haven, South Wales, UK. Maps were created in QGIS 3.10.3-A Coruña. Inset, geographic location of South Wales (red box). Purple star indicated Skomer marine nature reserve.

3.3.2 Laboratory regime

The following biometric data were recorded for each specimen; wet weight (g), length (mm), width (mm), and position within limpet stack. The sex of the individual was also determined, and assigned as either male (presence of developed penis), female (absence of penis), or transitionary (reduced penis) as described by (Coe, 1936; Figure 3.2). Haemolymph was collected from each sample by first carefully removing the flesh from the shell using a bunt-ended probe and allowing the haemolymph to pool in the shell cavity (Figure 3.3). Liquid tissue (haemolymph) was plated on general (TSA +2% NaCl) and selective (TCBS) culture media for bacterial load quantification, processed for DNA extraction and PCR-based diagnosis (universal *Vibrio* primers; Vezzulli *et al.*, 2012), and solid tissues were examined using histology.

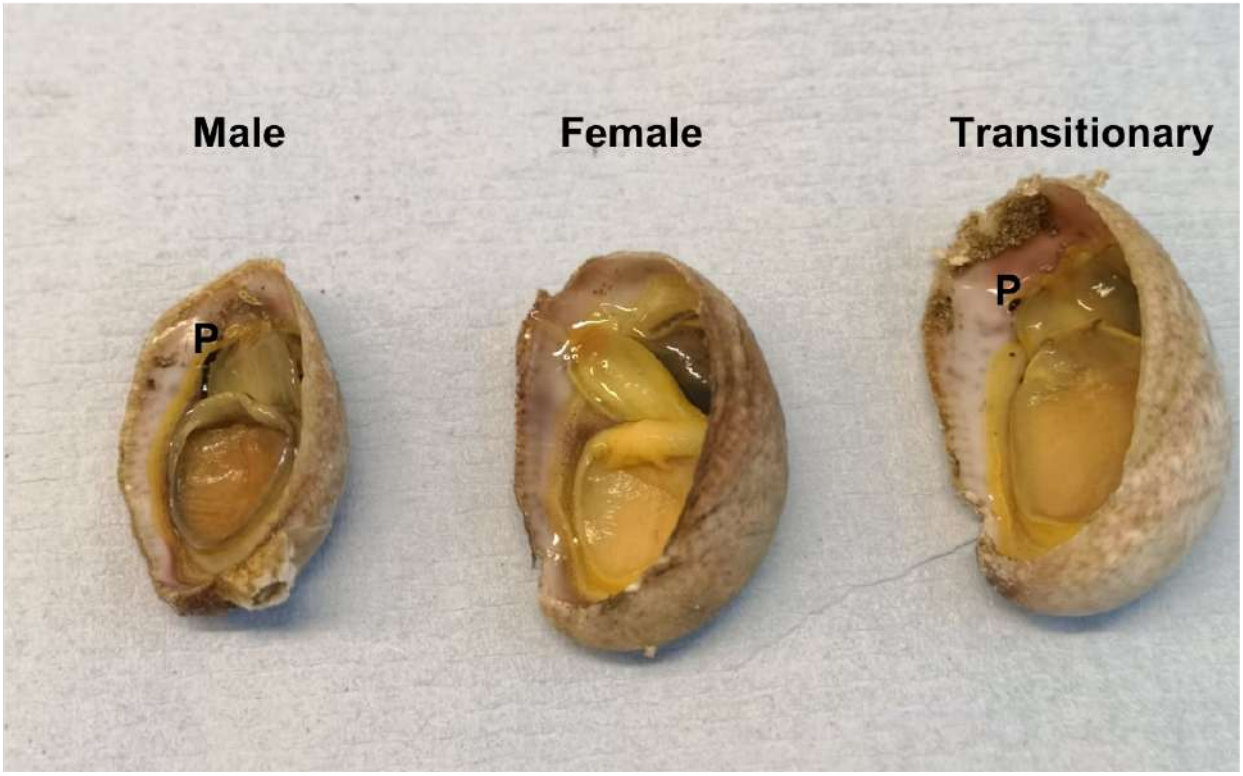

Figure 3.2: Male, female and transitionary *Crepidula. fornicata.* The presence of a fully formed penis (arrow pointing) indicates that the individual is male, the absence of penis indicates that the individual is female, and the presence of a reduced penis (black arrow) indicates that the individual is in a transitionary state.

Haemolymph was aspirated using a needle (22G) fitted onto a 1 mL sterile syringe. Total haemocyte counts (immune cells) were recorded using an improved Neubauer haemocytometer and optical microscope. Bacterial colony forming units (CFUs) were determined by spreading 100 uL of haemolymph diluted 1:100 with 3% NaCl solution on tryptone soya agar (TSA) plates supplemented with 2% NaCl (two (plates) technical replicates were performed per biological sample), and thiosulfate-citrate- bile salts-sucrose agar (TCBS). Plates were incubated at 25 °C for 24 h prior to counting CFUs. The haemolymph bacterial load is expressed as CFUs per mL haemolymph.

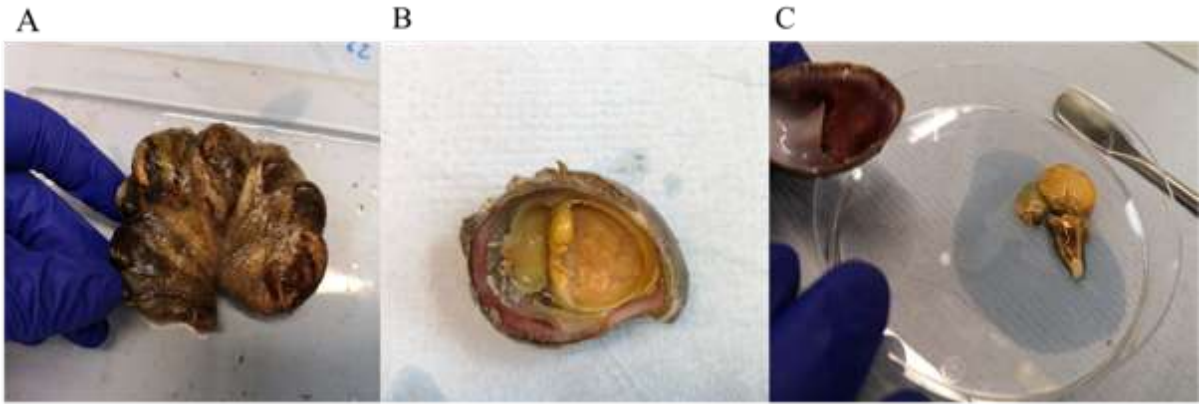

Figure 3.3: Processing *Crepidula fornicata* samples. (A) Full stack of *Crepidula. fornicata*. (B) Ventral surface of a limpet removed from the stack. (C) Detached tissue mass/flesh of limpet.

3.3.3 Tissue histology

Whole tissue histology was used to screen a subset (n = 343) of animals to visualise any potential immune responses to Vibrio-like bacteria, e.g. haemocyte infiltration or tissue damage. The process involved in carrying out whole tissue histology has been described in Chapter 2.3.8.

3.3.4 Molecular diagnostic techniques

3.3.4.1 DNA extraction

A range of commercially available DNA extraction kits namely the Sigma Aldrich GenElute ™ Blood Genomic DNA Kit, Qiagen DNeasy Blood and Tissue Kit, Qiagen DNeasy Powersoil Kit, the Omega Bio-TEK, E.Z.N.A® Tissue Kit, along with Chelex extraction, were initially trialled in order to extract DNA from the haemolymph of *C. fornicata* . DNA yields were quantified using the Invitrogen Qubit Fluorometer in combination with the Invitrogen dsDNA HS Assay Kit. The Sigma Aldrich GenElute™ Blood Genomic DNA Kit was selected as the for use throughout the sampling period based on DNA yields. Genomic DNA from *C. fornicata* haemolymph (100 µL) was extracted following the supplier's guidelines. For 29/1800 specimens it was not possible to obtain 100 µL of haemolymph due to their size, instead, 20 mg of solid foot tissue was used.

3.3.4.2 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in 25 µL total reaction volumes using 2x Mastermix (New England Biolabs Inc., Ipswich, USA), 1.25 µL oligonucleotide primes (10 µM) synthesized by Eurofins (Ebersberg, Germany), 2 µL of genomic DNA, and performed in a PCR thermocycler (BioRad Laboratories Inc., Hemel Hempstead, UK.). For amplification of *Vibrio*, the Vib1-F (GGCGTAAAGCGCATGCAGG) and Vib2-R (GAAATTCTACCCCCCTCTACAG) universal primer set (Vezzulli *et al*., 2012) was used and followed conditions described originally by Thompson *et al*. (2004) and modified by Vezzulli *et al.,* (2012). The expected amplicon size was 113 bp. Negative controls consisted of DEPC-Treated Molecular Biology Grade Water (Sigma Aldrich) in the absence of DNA template to avoid false positives due to contamination. Positive controls consisted of 1 µL DNA purified from the haemolymph of an infected donor crab. PCR products were visualised using 2 % (w/v) agarose/TBE gels stained with 3 µL Greensafe premium nucleic acid stain (NZYTech, Lisboa, Portugal). TBE gels consisted of 100 mL 1x TBE buffer, and 2 g agarose. Each gel was run at 100 volts for 45 minutes in TBE buffer.

3.3.4.3 Direct sanger sequencing

In preparation for sequencing positive signals, the amplicons were purified using HT ExoSAP-IT™ Fast high-throughput PCR product clean-up (Thermo Fisher Scientific, Altrincham, UK). Direct Sanger sequencing was carried out by Eurofins. In total, 72 samples were chosen at random for sequencing – distributed across both sites and from each month sampled. Of the 72 samples sent for sequencing, 22 of these returned usable data. Chromatograms were manually checked for mis-calls to ensure the accuracy of the nucleotides. The generated sequences were trimmed manually of primer regions and matched against the National Center for Biotechnology Information (NCBI) nucleotide database using BLASTn (Basic Local Alignment Search

Tool). Query Coverage (QC), Maximum Identity (MI) and E-value data were recorded for the top three returns per sample. Sequences were submitted to the NCBI short read archive (SRA) under accession numbers SRR13165025 – SRR13165046.

3.3.4.4 Phylogenetic analysis

The retrieved sequences were searched against GenBank using default BLASTn settings followed by restricting the search to the genus *Vibrio*. Bacterial nucleotides derived from *C. fornicata* were added to a selection of known, geographically distributed *Vibrio* reference sequences to make up a comprehensive dataset. A complete sequence alignment was achieved using the Clustal tool in MEGA X. Evolutionary analyses and reconstructions were carried out in MEGA X (Kumar *et al.*, 2018) using the maximum likelihood routine based on the Kimura 2-parameter model and an independent Neighbour Joining routine. A consensus tree with the highest log likelihood value (−150.41) from 1,000 bootstrap re-samplings was presented using iTOL software (Letunic and Bork, 2019).

3.3.5 Statistical analyses

Sample size calculations using an α-value of 0.05 and desired power >80% indicated that a minimum of 57 (1-sided test) up to 73 (2-sided test) limpets were required based on an *a priori* prediction of 15% (p1) to 35% (p2) prevalence of 'diseased' animals (in line with observations made by Le Cam and Viard (2011) when screening limpets for *C. celata*). In order to determine if specific predictor variables had a significant effect on the probability of finding *Vibrio*-positive limpets, binomial logistic regression models were carried out using the Logit link functions found in the MASS library (following Bernoulli distributions). Logistic models were carried out in RStudio v 1.2.5033 and R v 4.0.2. An information theoretical approach was utilised for both model selection and assessment of model performance (Richards, 2005). Initial models will from herein be referred to as full models. Using the drop1 function, each non-significant predictor variable from the full model was removed sequentially to increase the predictive

power of the final model. The function of drop1 is to compare the initial full model with the same model, with the least significant predictor variable removed. If the difference between the reduced model and initial full model is significant, the removed predictor variable is kept out of the new, reduced model. A Chi-square test is used for comparison of the residual sum of squares in both models in the case of binomial response variables. Variables included in the full model: *Vibrio* (PCR negative/positive, 0 or 1), location (Milford Haven or Swansea Bay), season (Spring (Mar, Apr, May), Summer (Jun, Jul, Aug), Autumn (Sep, Oct, Nov), Winter (Nov, Dec, Jan), sex (male, female, transitionary), wet weight (continuous number), position in stack (e.g., 1st, 2nd, 3rd, etc.). The critical value was 0.05 to accept a statistically significant result. All other statistics (tests of normality, Kruskal- Wallis, Mann-Whitney, Chi-square) were produced using GraphPad Prism v.8 for Mac (GraphPad Software, La Jolla California USA). Figures were prepared in GraphPad PRISM v8.

3.4 Results

3.4.1 Morphometric data

In total, 1,800 animals were surveyed (75 per month, per site). Across the three morphometric data types measured, i.e., wet weight (g), length (mm), and width (mm), individuals collected from Swansea Bay were significantly larger (P<0.0001, Mann-Whitney; Figure 3.4). The whole wet weight of *C. fornicata* collected from Milford Haven was found to be 7.99 ± 0.10 g (range = 0.4 to 20.17 g), while in Swansea Bay it measured 18.86 ± 0.3 g (range = 0.29 to 70.1 g). Length of *C. fornicata* from Milford Haven was found to be 36.06 ± 0.18 mm (range = 1 to 53 mm), while in Swansea Bay it was 46.49 ± 0.26 mm (range = 14 to 61 mm). Shell width of *C. fornicata* from Milford Haven was measured at 23.91 ± 0.1 mm (range =11 to 43 mm), while in Swansea Bay shell length was 27.83 ± 0.14 mm (range = 11 to 42 mm).

At both Milford Haven and Swansea Bay, a similar pattern was observed with regards to the numbers of males, females, and transitionary individuals that formed the

population. At both sites, a significant difference in the prevalence of males, females, and transitionary *C. fornicata* was observed (X^2(2, n=1800)=8.852., P = 0.012; Chi-square). Female *C. fornicata* formed the majority of the population structure. At Milford Haven, females constituted 48%, males 25%, and transitionary 27% of *C. fornicata* sampled. At Swansea Bay, females made up 49%, males 20%, and transitionary 31% of the population (Figure 3.5). In total 895 stacks were collected during the sample period. In the 268 stacks out of the 276 (97%) cases where a male was identified within the stack, a male *Crepidula fornicata* was found to be on the top of the stack. If no male was found to be present within the stack, and the stack consisted only of female and transitionary individuals, a transitionary individual was found to be on top of the stack in 63% (67/107) cases.

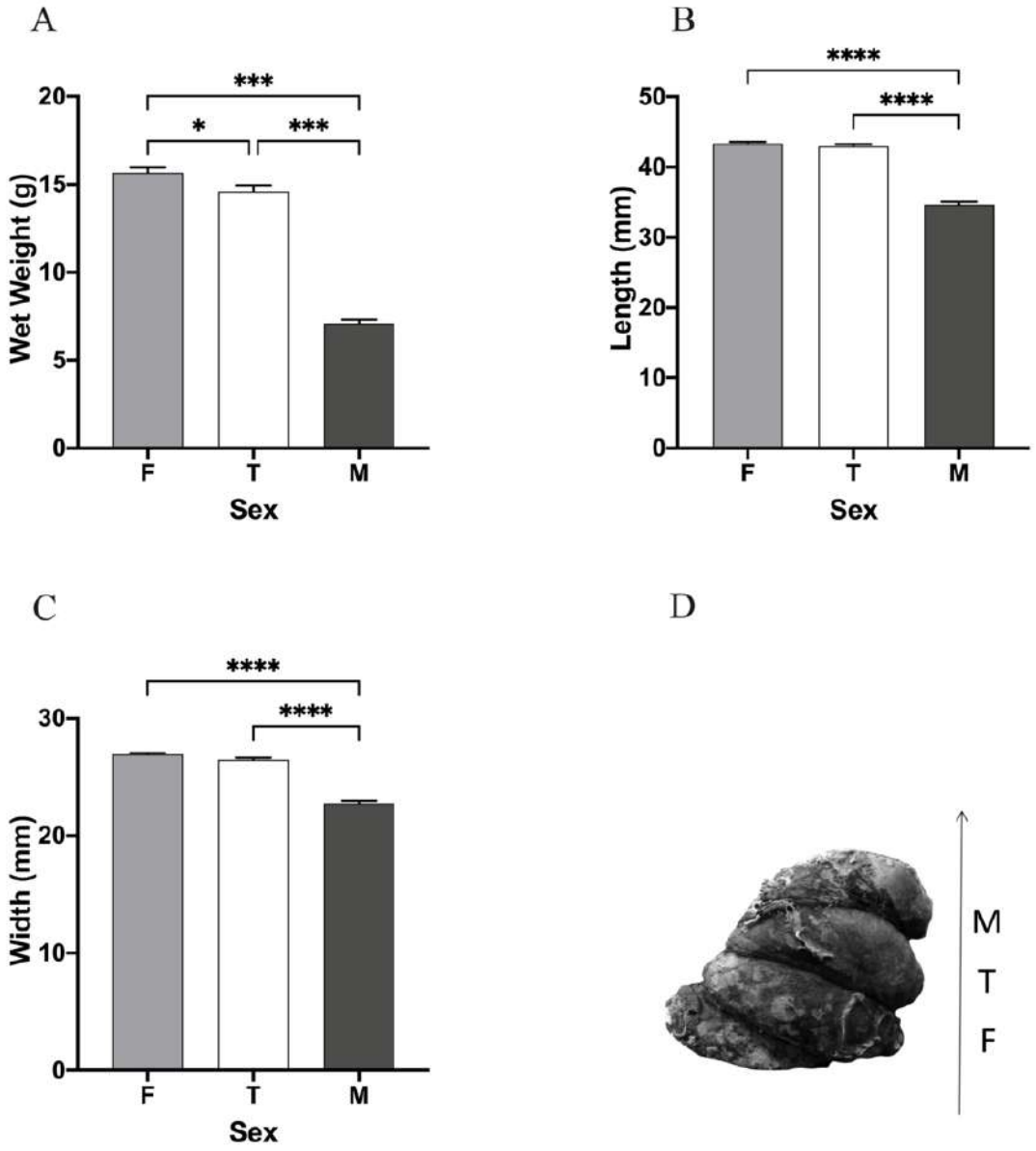

Figure 3.4: Morphometric measurements of *Crepidula fornicata* collected at Milford Haven (MH; n=900), and Swansea Bay (SB; n=900). (A) Wet weight (g), (B) Length (mm), (C) Width (mm). Asterisk denotes significant difference ($P < 0.0001$). (D) Generally accepted position of female, transitionary, and male individuals within a stack.

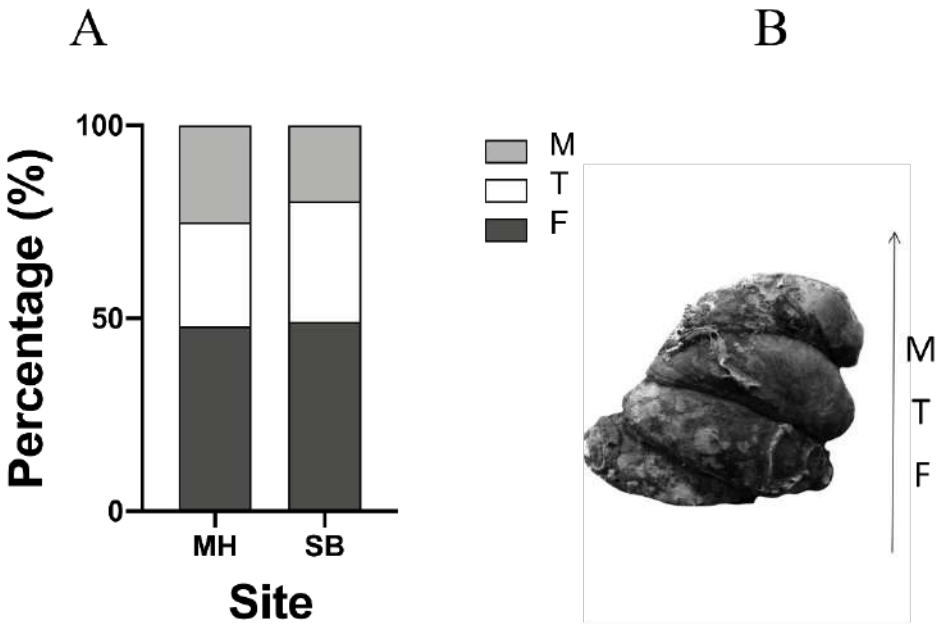

Figure 3.5: Proportion of male, female, and transitionary states of *Crepidula fornicata* sampled across Milford Haven (n=900), and Swansea Bay (n = 900) from Jan 19-Dec 19. (D) Generally accepted position of female, transitionary, and male individuals within a stack.

Female and transitionary *Crepidula fornicata* were found to be significantly heavier than males (P<0.01, Kruskal-Wallis; Figure 3.6), females were also found to be slightly heavier than transitionary individuals (P =.013). Wet weight of females was 15.67 ± 0.29 g (range = 2.69 to 70.10 g), males weighed 7.06 ± 0.26 g (range = 0.29 to 41.34 g), and transitionary 14.58 ± 0.37 g (range = 0.57 to 39.61 g). Females and transitionary animals were found to be significantly larger in terms of length and width compared to males (P<0.001, Kruskal-Wallis). Females length measured 43.35 ± 0.24 mm (range = 22 to 60 mm), males length measured 34.66 ± 0.39 mm (range = 14 to 58 mm), and transitionary length measured 42.62 ± 0.35 mm (range =10 to 61 mm). Females width measured 26.93 ± 0.12 mm (range = 14 to 43 mm) males measured 22.76 ± 0.2 mm (range= 11 to 41 mm) and transitionary measured 26.48 ± 0.17 mm (range =13 to 42 mm).

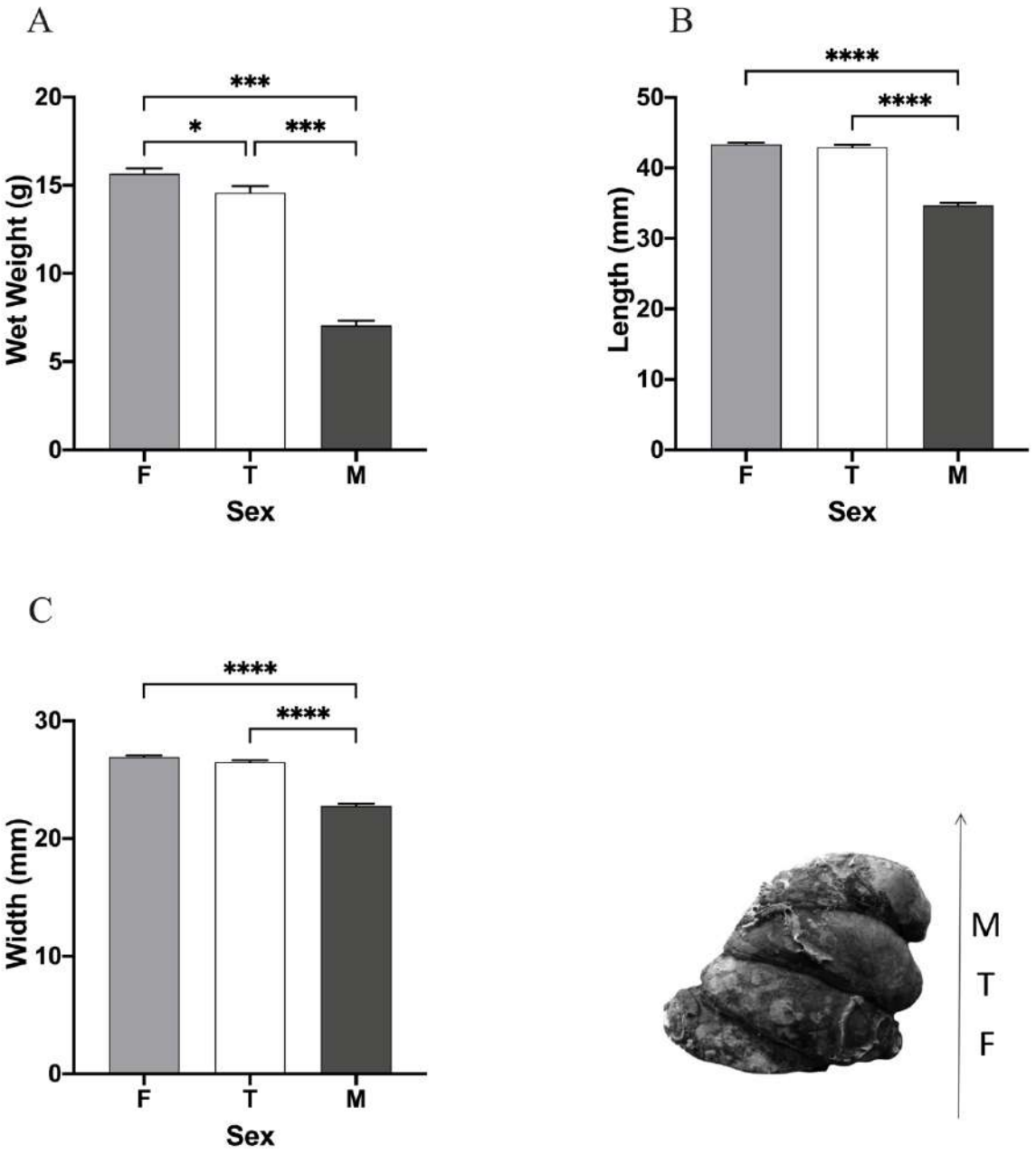

Figure 3.6: Size distribution of Male (M), Female (F), and Transitionary (T) *Crepidula fornicata* surveyed across Swansea Bay and Milford Haven from Jan 19 – Dec 19 (n=1800). (A) Wet weight (g) (B) Length (mm) (C) Width (mm) (D) Generally accepted stack layout i.e. female at the bottom of the stack, with transitional individuals in the middle of the stack, and males closer to the top.

3.4.2 Bacterial presence

Haemolymph was retrieved from 1,780 individuals and plated on the generalist agar TSA (+ 2% NaCl), and on the *Vibrio* selective TCBS (Figure 3.7). Of these, <1% of individuals contained no cultivable bacterial colonies on either TSA (n = 8) or TCBS (n = 12) media. Bacterial growth on TSA was 2.1 ± 0.071 x 10^5 CFU/ml CFU/mL (range = 0 to 3.8 x10^6 CFU/mL), whilst on TCBS it was 1.8 ± 0.066 x10^5 CFU/ mL (range = 0 to 2.69 x10^6 CFU/mL) No significant differences in CFU numbers from the haemolymph of *C. fornicata* between Milford Haven and Swansea Bay on TSA (*P* = 0.487 Mann-Whitney) or TCBS (*P* = 0.665) were observed.

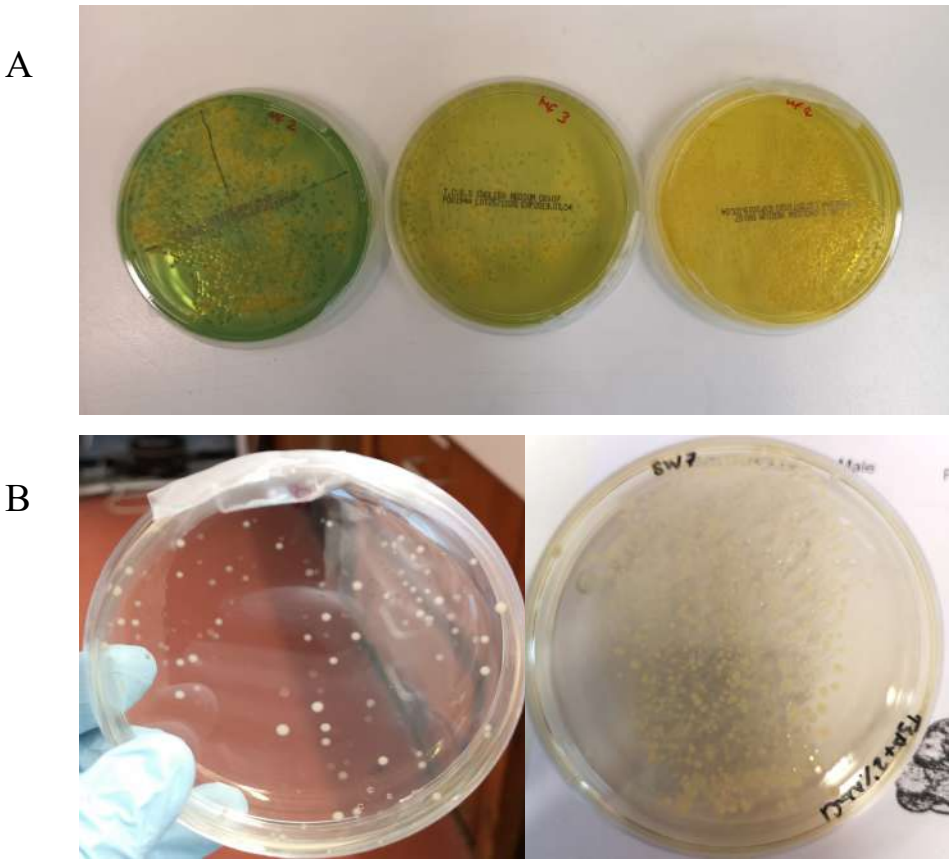

Figure 3.7: Representative growth of cultivable bacteria from haemolymph of *Crepidula fornicata* incubated at 25 °C for 24 h on (A) TCBS - Vibrio selective agar, the yellow colour change results due to fermentation of sucrose in the medium, bacteria not capable of fermenting sucrose produce green to blue-green colonies (B) Generalist TSA agar.

A significant temporal effect was observed in CFU numbers present in the haemolymph of *C. fornicata* found at each site between January 2019 and December

2019 (Figure 3.8). This trend was seen on CFU growth on both TSA (P<0.0001; Kruskal-Wallis) and TCBS (P<0.001). Culturable bacterial numbers in the haemolymph of *C. fornicata* were at their highest during the summer season (Table 3.1). This was observed on both TSA, and TCBS agar, 4.09 ± .176 $\times 10^5$ CFU/mL (range = 3.5 $\times 10^3$ to 2.61 $\times 10^6$ CFU/mL), and 3.99 ± .188 $\times 10^5$ CFU/mL (range = 0 to 2.7 $\times 10^6$ CFU/mL), respectively. Conversely bacterial numbers were at their lowest during the winter season on both TSA, and TCBS agar, 1.17 ± .0773 $\times 10^5$ CFU/mL (range = 0 to 1.14 $\times 10^6$ CFU/mL), and 7.3 ± .674 $\times 10^4$ CFU/mL (range = 5 $\times 10^2$ to 7 $\times 10^5$ CFU/mL) respectively.

Table 3.1: CFU counts on TSA and TCBS media from Milford Haven and Swansea Bay, across Spring, Summer, Autumn, and Winter.

Agar	Season	Min	Max	Mean	SE
TSA	Spring	0	3.87 $\times 10^6$	1.98 $\times 10^5$	1.42 $\times 10^4$
	Summer	3.5 $\times 10^3$	2.61 $\times 10^6$	4.09$\times 10^5$	1.76 $\times 10^4$
	Autumn	0	2.22 $\times 10^6$	1.24 $\times 10^5$	9.75 $\times 10^3$
	Winter	0	1.14$\times 10^6$	1.17 $\times 10^5$	7.73 $\times 10^3$
TCBS	Spring	0	1.07 $\times 10^6$	1.46 $\times 10^5$	9.64$\times 10^3$
	Summer	0	2.69 $\times 10^6$	3.99 $\times 10^5$	1.88 $\times 10^4$
	Autumn	0	8.12 $\times 10^5$	1.04 $\times 10^5$	7.42 $\times 10^3$
	Winter	5.0 $\times 10^2$	7.00 $\times 10^5$	7.33 $\times 10^4$	6.74 $\times 10^3$

CFU counts on both TSA, and TCBS agar peaked during August, 6.18$\times 10^5$ ± 2.63 $\times 10^4$ CFU/mL (range = 3.75 $\times 10^4$ to 2.08 $\times 10^6$ CFU/mL), and 7.07 $\times 10^5$ ± 3.35 $\times 10^4$ CFU/mL (range = 3 $\times 10^4$ to 2.16 $\times 10^4$ CFU/mL) respectively. CFU counts on TSA agar were lowest during November, 7.29 $\times 10^4$ ± 9.82 $\times 10^3$ CFU/mL (range = 4 $\times 10^3$ to 7 $\times 10^5$ CFU/mL). Growth on TCBS agar was at its lowest during December, 3.56 $\times 10^4$ ± 5.87 $\times 10^3$ CFU/mL (range = 5 $\times 10^2$ to 4.48 $\times 10^5$ CFU/mL).

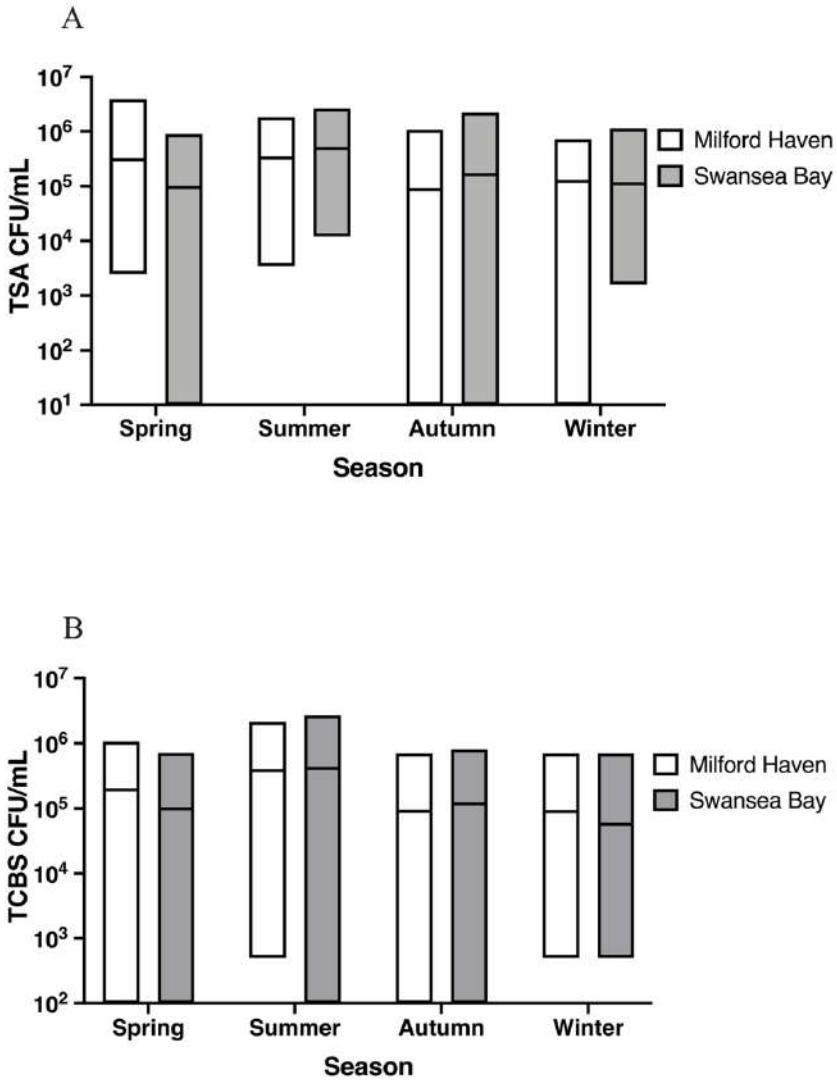

Figure 3.8: Temporal changes in the pattern of cultivable colony-forming units (CFU/mL) from the haemolymph of *Crepidula fornicata* (n=1780). (A) Bacterial CFU growth on TSA (B) TCBS media across seasons spring, summer, autumn, and winter. Individuals were sampled from Swansea Bay and Milford Haven. *Crepidula fornicata* haemolymph was collected, diluted 1:100 with sterile 3% saline, and 100 uL streaked on the respective agar plates, incubated at 25 °C for 24 h prior to counting CFUs. The haemolymph bacterial load is expressed as CFUs per mL haemolymph

3.4.3 Total haemocyte counts

Freely circulating haemocyte numbers were quantified from a total of 1,771 individuals (Milford Haven n = 883, Swansea Bay n = 888). Overall, significantly fewer haemocytes were present in the haemolymph of limpets from Milford Haven when compared to Swansea Bay (P<0.01; Mann-Whitney). Haemocyte counts from *C. fornicata* sampled at

Milford Haven were found to be 2.7 $x10^6 \pm 9.3\ x10^4$ HC /mL (range = $1.3\ x10^5$ to $3.3\ x10^7$ HC /mL). Haemocyte counts from individuals sampled at Swansea Bay were found to be $3.3\ x10^6 \pm 1.1\ x10^5$ HC /mL (range = $3\ x10^5$ to $3.1\ x10^7$ HC /mL). No relationship was found between the size of the individual and the number of haemocytes found within the haemolymph sample (P = .380; Spearman).

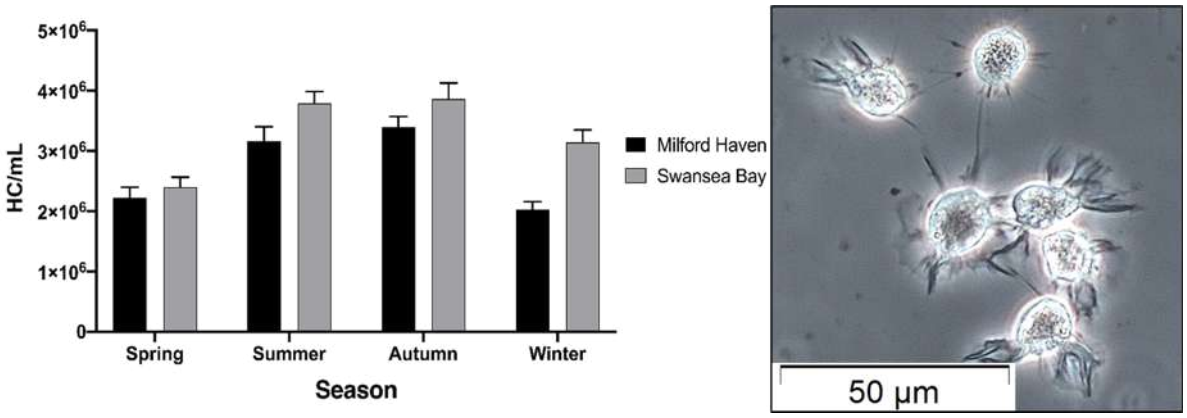

Figure 3.9: Temporal changes in the pattern of total haemocyte counts (HC/mL) observed in the haemolymph of *Crepidula fornicata*. Individuals were collected from Milford Haven (n=883), and Swansea Bay (n=888), across Spring, Summer, Autumn, and Winter. Inset, contrast image depicting the typical appearance of haemocytes (granular, motile).

A significant temporal effect was observed with haemocyte counts between seasons (P<0.0001; Kruskal-Wallis; Figure 3.9). Limpets collected during autumn had the highest haemocyte counts, at $3.62 \times10^6 \pm 1.6 \times10^5$ HC/mL (range = 4.05×10^5 to 3.1×10^7 HC/mL). Summer had haemocyte counts of $3.48 \times10^6 \pm 1.53 \times10^5$ HC/mL (range = 2.8×10^5 to 3.311×10^7 HC/mL). Haemocyte counts during winter were $2.59 \times10^6 \pm 1.25 \times10^5$ HC/mL (range = 2.1×10^5 to 1.44×10^7 HC/mL). Spring had the lowest haemocyte counts, of $2.31 \times10^6 \pm 1.2 \times10^5$; (range =$1.3 \times10^5$ to 1.64×10^7 HC/mL). March was found to be the month with the lowest haemocyte counts of $1.79 \times10^6 \pm 1.75 \times10^5$ HC/mL (range = 1.3×10^5 to 1.47×10^7 HC/mL). Haemocyte counts reached their peak in September at $4.64 \times10^6 \pm 3.91 \times10^5$ HC/mL (range = 5.1×10^5 to 3.1×10^7 HC/mL).

3.4.4 Stack size

Between Milford Haven and Swansea Bay a significant difference in stack size i.e., how many individuals made up a stack (Figure 3.10), was observed, with stacks at Swansea Bay consisting of more individuals than those at Milford Haven ($X^2(10, N=1800) = 95.0$, $P<0.0001$; Chi-square test). At Milford Haven, stack size ranged from 1 to 5 individuals, and at Swansea Bay, stack size ranged from 1 to 11 individuals. At Milford Haven, 50% of stacks consisted of just one individual, 35% of stacks consisted of 2 individuals, 12% stacks of 3 individuals, and the remaining 3% was distributed between stacks of 4 and 5. At Swansea Bay 40% of stacks consisted of stacks of 1, 25% consisted of stacks of 2, 13% consisted of stacks of 3.8% of stacks of 4, and the remaining 14% was distributed among stacks of 5 to 11 individuals. A temporal effect was observed on the number of *C. fornicata* that formed stacks ($P < 0.0001$; Kruskal-Wallis). Stack size was smallest during the summer, ranging from 1 to 7 limpets (n = 450) per stack. Stack size was largest during winter ranging from 1 to 11 (n = 440) per stack.

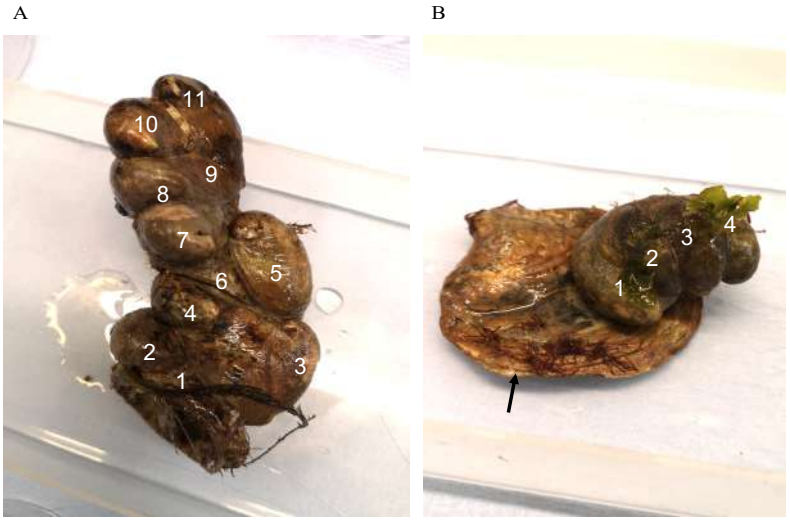

Figure 3.10: Examples of stack sizes of slipper limpets observed over the course of the sampling period from Jan 19 - Dec 19 (A) The largest stack sampled was found at Swansea Bay, Jan 19, with 11 individuals counted. (B) A stack that closer represents the average stacks collected over the sampling period (n=3), this stack had also settled on the empty shell of the native oyster *Ostrea edulis* (arrow).

3.4.5 Relationship(s) between position within a stack and haemolymph measurements

The influence of within stack position was explored to look at its potential impact on morphometric measurements of wet weight (g), length (mm), and width (mm). Within stack position was found to have an effect on the wet weight of the individual, with individuals at the bottom of the stack being larger in size than those higher up in the stack (P<0.0001, Kruskal-Wallis) (Figure 3.11).

The impact of where in a stack an individual was located was also investigated to determine if this contributed to variation in CFU counts on TSA and TCBS media, or haemocyte counts (Figure 3.12). Within stack position was found to not have a significant effect on CFU counts on TSA (P=0.32) or TCBS (P=0.063). Within stack position was found to have a slight effect on haemocyte counts (P=0.0453). Initially, data from Milford Haven, and Swansea Bay were combined. This effect was only observed between individuals in position 1 and position 6. The data were then separated by site, as only stacks at Swansea Bay exceeded a stack size of 5 individuals. When separated by site, this slight significant effect was no longer observed, at either Milford Haven (P=0.06; Kruskal-Wallis) or at Swansea Bay (P=0.31; Kruskal-Wallis).

The ratio of bacterial colony forming units (CFUs) to haemocyte counts (HCs), and how this was impacted on by within stack position was also considered. Within stack position did not significantly influence the ratio of colony forming bacterial units to haemocyte counts (P=0.3457; Kruskal-Wallis).

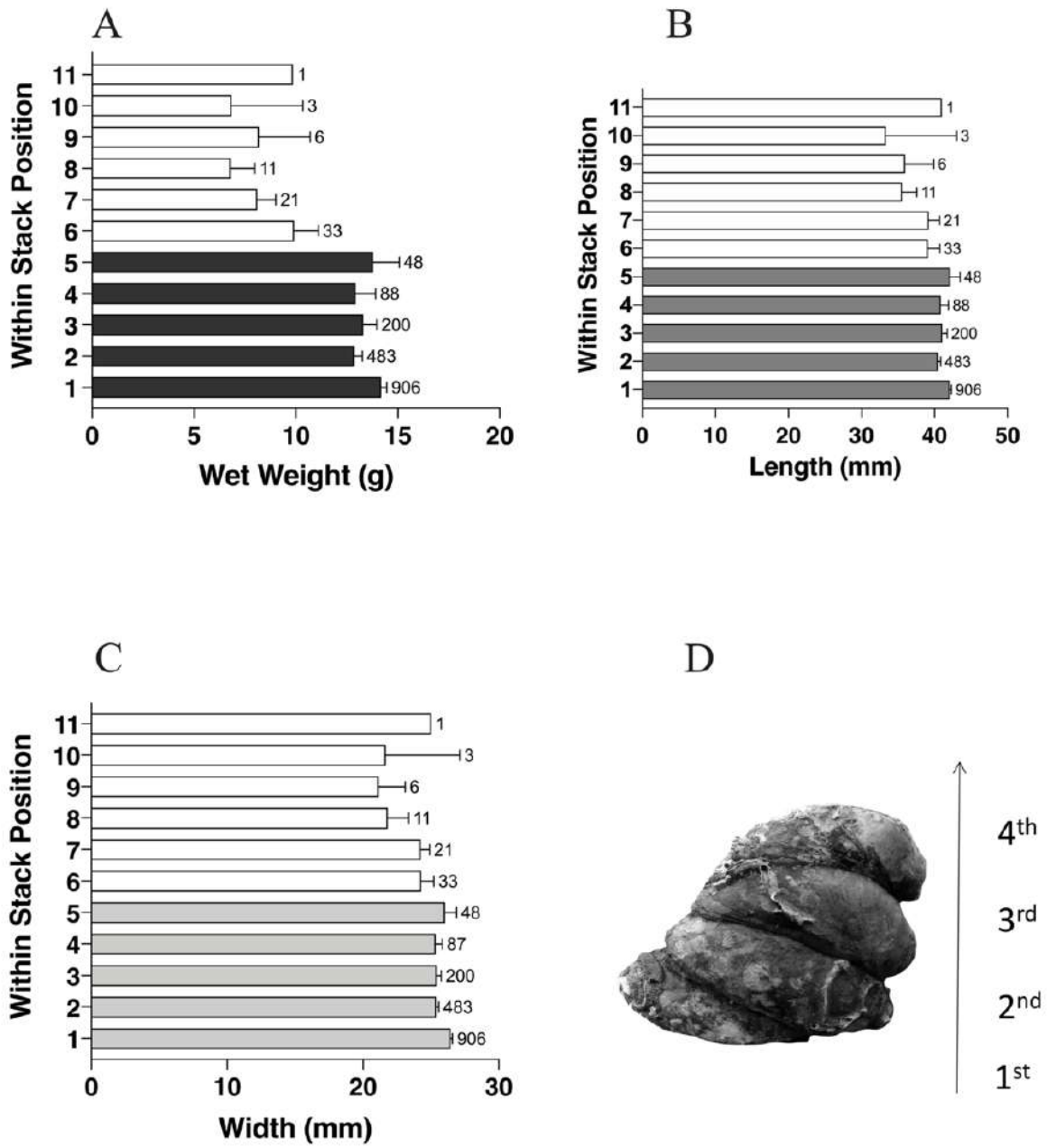

Figure 3.11: Relationship between stack position and morphometric measurements of *Crepidula fornicata* (n=1800). (A) wet weight (g), (B) length (mm), and (C) width (mm). (D) Example of *Crepidula fornicata* stack with multiple individuals and labelling system used. Within stack position 1-5 consists of Milford Haven and Swansea Bay combined. Within Stack position 6-11 is Swansea Bay only (coloured in white).

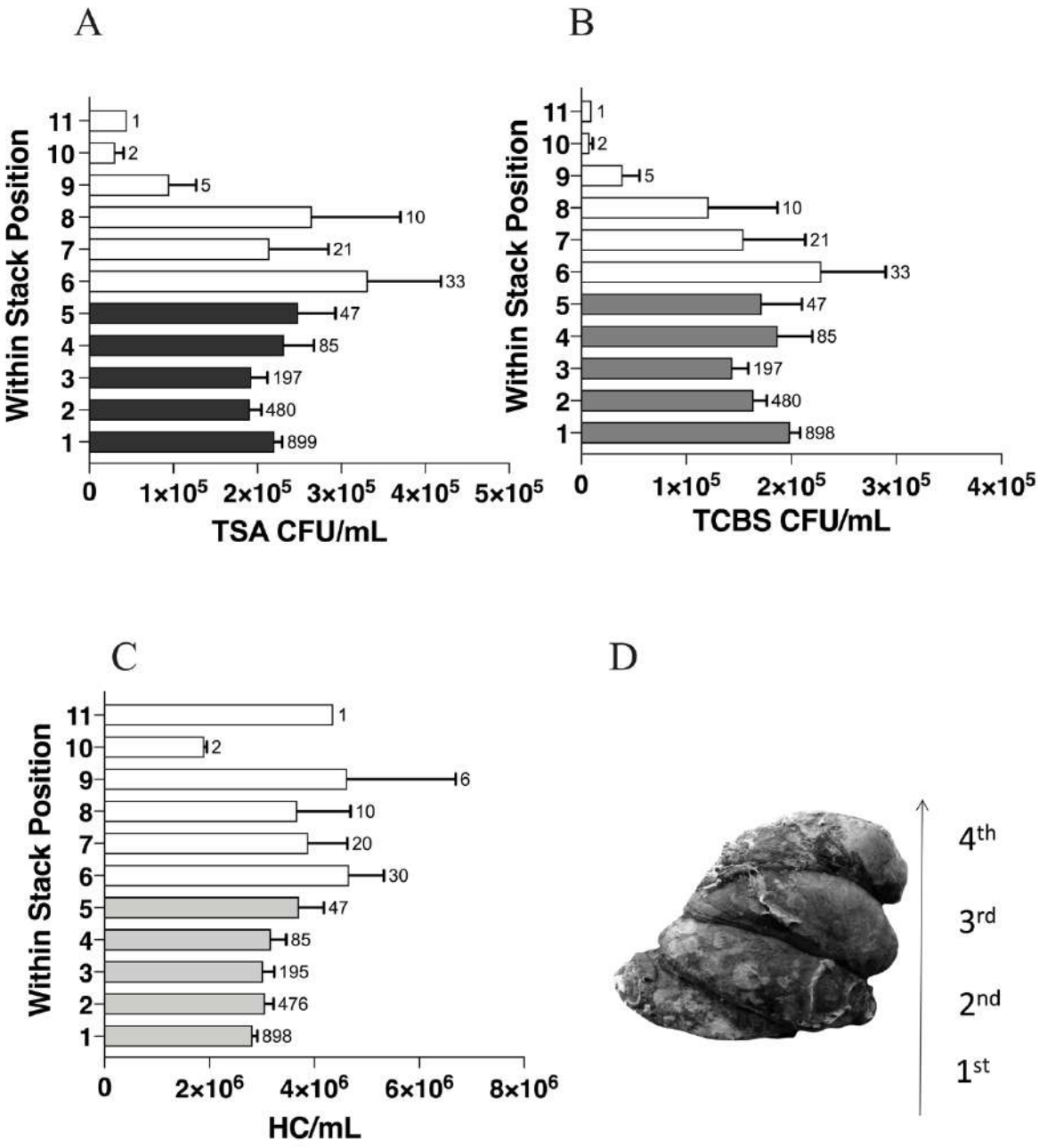

Figure 3.12: Influence of stack position on bacterial colony forming units (n=1780), and haemocyte counts (n=1730) in the haemolymph of *Crepidula fornicata*. (A) TSA CFU/mL numbers (B) TCBS CFU/mL numbers (C) Total Haemocyte Numbers (D) Example of *Crepidula fornicata* stack with multiple individuals and the subsequent labelling system employed. Within stack position 1-5 consists of Milford Haven and Swansea Bay combined. Within Stack position 6-11 is Swansea Bay only (coloured in white).

3.4.6 Molecular screen for Vibrio-like bacteria

All 1,800 limpets collected from the yearlong survey, 900 from Milford Haven and 900 from Swansea Bay were screened for the presence of *Vibrio* through PCR and agarose gel electrophoresis (Figure 3.13). Of these limpets overall 83% tested positive through PCR detection. It was found that 85% (763/900) of limpets in Swansea Bay, and 81% (729/900) of samples from Milford Haven tested positive through PCR (Figure 3.14). August was the month with the highest number of PCR positive samples with 150 (150/150; 100%), and February with the lowest number of PCR positives with 84 (84/150; 56%).

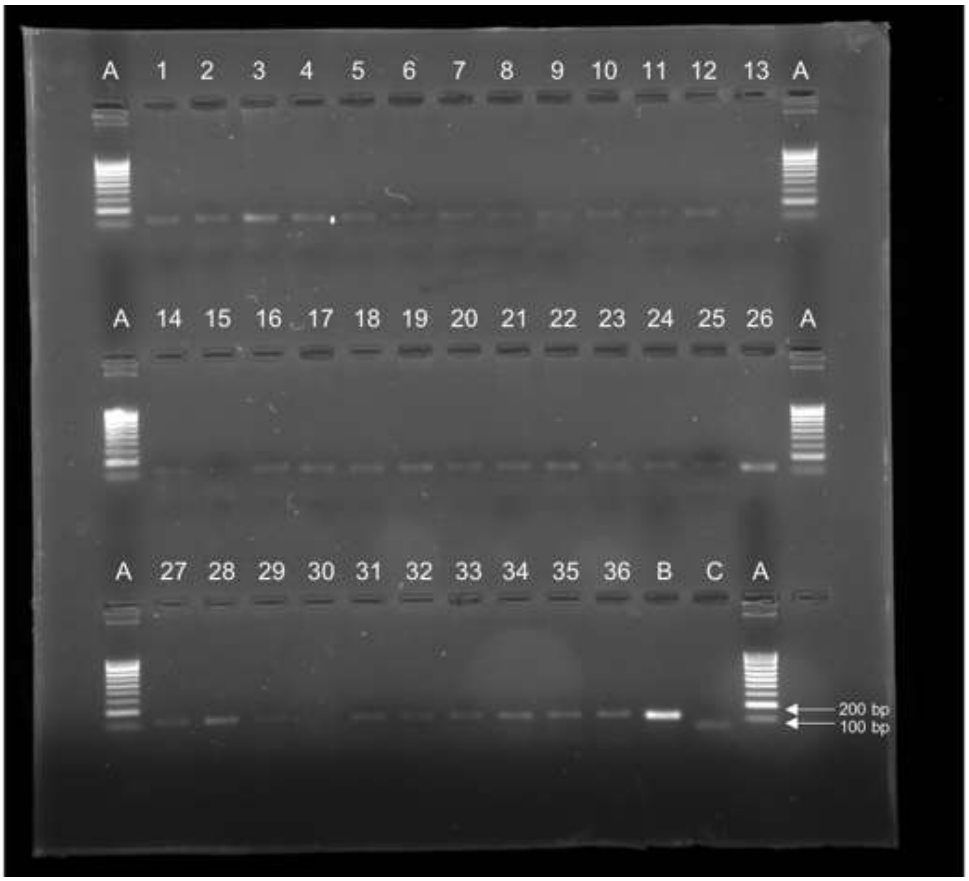

Figure 3.13: Agarose gel electrophoresis of PCR amplified products targeting *Vibrio* sequences (16s V6 hyper variable region). (A) Ladder (NZYTech, Lisboa, Portugal), (B) Positive control consisted of 1 µL DNA purified from the haemolymph of an infected donor (C) Negative control (DEPC-treated ultra-pure water). Wells 1-14, 16-29, and 31-36 are positive bands indicating amplification of *Vibrio* products in *Crepidula fornicata* sampled from Milford Haven in November 2019.

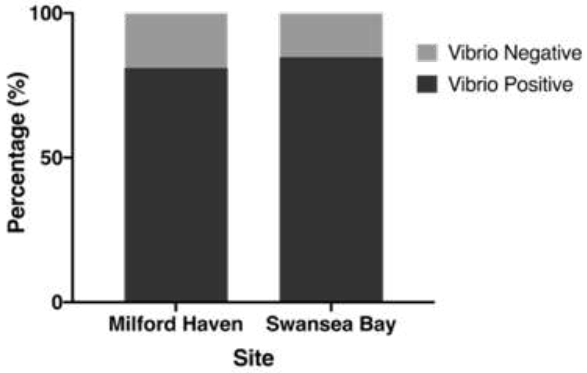

Figure 3.14: Prevalence of Vibrio-like bacteria at each site determined via PCR-based screening. Haemolymph samples were used for 1771/1800 individuals, solid tissue samples were used for 29/1800 due to their small size. Of the 29 tissue samples used, 14 were PCR positive for Vibrio-like bacteria. Also, of the 29 tissue samples used, the haemolymph of 12 of these individuals were plated on TSA and TCBS agar and grew cultivable bacteria.

3.4.7 Biometric and environmental variables associated with *Vibrio* occurrences in *Crepidula fornicata*

All biometric data recorded during the processing of *Crepidula fornicata* individuals' (size, sex, position in stack), along with collection site, CFU counts, and haemocyte counts were included in the initial full model to determine the factors that may contribute to the presence of *Vibrio* within the samples.

Model 1 combined data from both sites, with *Vibrio* as the response variable (Table 3.2). It was found that season, size (wet weight), and CFU growth on TCBS agar were significant factors associated with the presence of *Vibrio*. A significant decrease in the number of *Vibrio* positives was observed during Spring (P = 0.00539), and Winter (P = 0.0064). Larger *Crepidula* were more likely to be *Vibrio* positive when compared with *Vibrio* negative individuals (P = 0.00106, mean 13.84 ± 8.75 g; mean ± S.D vs 11.44 ± 7.8 g; mean ± SD, respectively). Individuals with increased growth on TCBS agar were more likely to test positive through PCR for *Vibrio* than those individuals who tested negative for *Vibrio* (P < 0.0001; 1.99 x10^5 ± 7.66x10^3 CFU/mL vs 9.02 x10^4 ± 8.73 x10^3 CFU/mL, respectively) (Figure 3.15).

A selection of *Vibrio*-positive amplicons (n = 72) from both sampling sites and each month were sent for direct sequence analysis using Sanger's method in a bid to identify putative *Vibrio* to species level (although the target region is small, 113 bp). Of these 72 samples, 22 usable sequences were generated with 6 proving clear matches to known *Vibrio* species, 1 matching to an unclassified *Vibrio* species, and 15 matching to uncultured bacteria (Table 3.3.)

Table 3.2: Binomial logistical regression model testing the influence of biometric and environmental predictor variables on the presence of Vibrio-like bacteria in *Crepidula fornicata* sampled from Swansea Bay and Milford Haven from Jan 19 – Dec 19

Model	Predictor Variable	Estimate Slope	SE	*P*-value
Full_Model ***Vibrio*** ~ Site + Season + Sex + Position.In.Stack + Wet.Weight + TSA + TCBS + Haemocyte.Counts	SiteSB	0.03827	0.1913	0.84142
	SeasonSpring	-0.5164	0.1878	0.00597**
	SeasonSummer	-0.07122	0.2272	0.7539
	SeasonWinter	-0.5723	0.1894	0.00251**
	SexM	-0.275	0.1955	0.15965
	SexT	-0.0008116	0.1575	0.99589
	Position.In.Stack	0.05505	0.05704	0.3345
	Wet.Weight	0.02108	0.01263	0.09492.
	TSA	4.455E-07	4.609E-07	0.3338
	TCBS	0.000001913	5.835E-07	0.00104**
	Haemocyte.Counts	1.53E-10	2.329E-08	0.99476
Model_reduced_1 ***Vibrio*** **~ Site + Season + Sex + Position.In.Stack +Wet.Weight + TSA+ TCBS**	SiteSB	2.23E-02	1.87E-01	0.905308
	SeasonSpring	-5.35E-01	1.83E-01	0.003544**
	SeasonSummer	-7.85E-02	2.25E-01	0.727346
	SeasonWinter	-5.52E-01	1.87E-01	0.003118**
	SexM	-2.47E-01	1.94E-01	0.201822
	SexT	6.90E-03	1.57E-01	0.964994

	Position.In.Stack	4.99E-02	5.64E-02	0.376824
	Wet.Weight	2.37E-02	1.25E-02	0.058003.
	TSA	4.25E-07	4.48E-07	0.3428
	TCBS	1.89E-06	5.69E-07	0.000879***
Model_reduced_2				
Vibrio **~ Season + Sex + Position.In.Stack + Wet.Weight + TSA + TCBS**	SeasonSpring	-5.29E-01	1.83E-01	0.00386**
	SeasonSummer	-7.09E-02	2.24E-01	0.752181
	SeasonWinter	-5.21E-01	1.85E-01	0.004756**
	Position.In.Stack	2.20E-02	4.47E-02	0.623254
	Wet.Weight	2.95E-02	8.79E-03	0.000781***
	TSA	4.05E-07	4.48E-07	0.366341
	TCBS	1.89E-06	5.70E-07	0.000903***
Model_reduced_3				
Vibrio **~ Season + Wet.Weight + TSA + TCBS**	SeasonSpring	-5.24E-01	1.83E-01	0.004149**
	SeasonSummer	-7.22E-02	2.25E-01	0.747685
	SeasonWinter	-5.02E-01	1.81E-01	0.005443**
	Wet.Weight	2.92E-02	8.75E-03	0.000854***
	TSA	4.15E-07	4.48E-07	0.353767
	TCBS	1.89E-06	5.70E-07	0.000903***
Model_reduced_4				
Vibrio **~ Season + Wet.Weight + TCBS**	SeasonSpring	-5.06E-01	1.82E-01	0.00539**
	SeasonSummer	-4.87E-02	2.23E-01	0.82692
	SeasonWinter	-4.92E-01	1.80E-01	0.0064**
	Wet.Weight	2.85E-02	8.70E-03	0.00106**
	TCBS	2.24E-06	4.45E-07	4.97E-07***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 # *Vibrio* = PCR positive

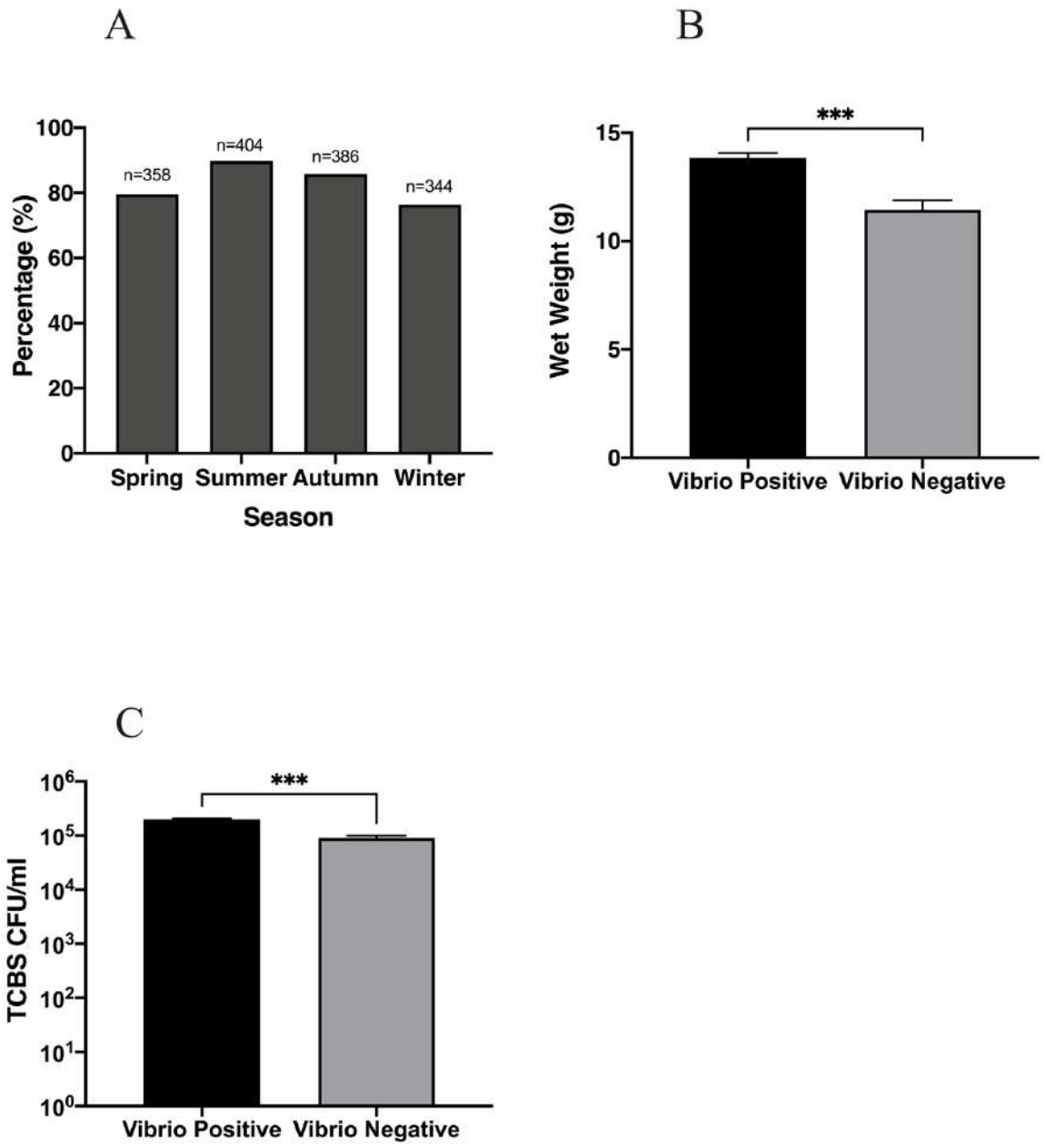

Figure 3.15: Significant variables associated with the detection of Vibrio in *Crepidula fornicata* individuals, determined through binomial logistical regression modelling. (A) Effect of season on percentage (%) of *C. fornicata* individuals that were PCR positive for Vibrio- like bacteria(n=1492). Individuals during the summer months were significantly more likely to test positive via PCR for Vibrio-like bacteria than during other seasons (B) Effect of size on the number of individuals that were PCR positive (n=1492) or PCR negative (n=308) for Vibrio-like bacteria. Larger individuals were significantly more likely to test positive for *Vibrio* overall. (C) *C. fornicata* individuals that were PCR positive (n=1480) for Vibrio-like bacteria had significantly more bacterial colony forming units per mL haemolymph compared with those that were PCR negative (n=299). Asterisk denotes significant differences ($P \leq 0.05$).

Table 3.3 BLASTn outputs from the 22 Vibrio-like sequences amplified via PCR from *Crepidula fornicata* haemolymph

Site	Month	Individual	Closest match	Accession number	QC(%)	% ID	Amplicon size	SRA submission ID
Jan	MH	13	Uncultured bacterium clone SanDiego_a2787	KF799598.1	72	97.78	61	SRR13165046
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	72	97.78		
Feb	MH	11	*Vibrio mediterranei*	HE584789.1	96	95.92	50	SRR13165045
			Vibrio sp. LiUU-B-16	DQ068946.1	92	95.74		
Feb	MH	11	*Vibrio* sp. IO3	HQ848040.1	82	100	64	SRR13165034
			Vibrionaceae bacterium DS3	EF584037.1	81	100		
Feb	MH	25	Uncultured bacterium clone SanDiego_a2787	KF799598.1	70	97.8	62	SRR13165034
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	70	97.8		
Feb	MH	25	*Vibrio tasmaniensis* isolate *25*	EF178480.1	85	98.15	63	SRR13165030
			Vibrio toranzoniae strain 2-2	MT510186.1	85	96.3		
Sep	MH	39	*Vibrio toranzoniae* strain 2-2	MT510186.1	86	100	47	SRR13165029
			Vibrio crassostreae strain 1-15	MT510175.1	100	100		
Oct	MH	43	Uncultured bacterium clone SanDiego_a2787	KF799598.1	63	97.67	66	SRR13165028
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	63	97.67		
Oct	MH	47	Uncultured bacterium clone SanDiego_a2787	KF799598.1	95	93.65	66	SRR13165027
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	95	93.65		

Nov	MH	55	Uncultured bacterium clone DLPYS_MD02_081	KC852641.1	100	97.73	44	SRR13165026
			Uncultured bacterium clone SanDiego_a3077	KF799522.1	100	95.45		
Dec	MH	17	Uncultured bacterium clone SanDiego_a2787	KF799598.1	72	100	62	SRR13165025
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	72	100		
Dec	MH	17	*Vibrio tasmaniensis* isolate 25	EF178480.1	92.4	98.11	53	SRR13165044
			Vibrio toranzoniae strain 2-2	MT510186.1	87.8	100		
Dec	MH	42	Uncultured bacterium clone SanDiego_a2787	KF799598.1	87	91.38	62	SRR13165043
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	87	91.38		
Dec	MH	42	*Vibrio celticus* strain Rd 8.15	NR_116066.1	90	100	60	SRR13165042
			Vibrio sp. IO3	HQ848040.1	93	98.21		
Jan	SB	21	Uncultured bacterium clone SanDiego_a2787	KF799598.1	100	97.78	44	SRR13165041
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	100	97.78		
Jan	SB	21	*Vibrio kanaloae* strain DS1904-Y1124	MT269633.1	100	100	49	SRR13165040
			Vibrio gallaecicus strain DS1904-Y1112	MT269632.1	100	100		
Jan	SB	52	Uncultured bacterium clone SanDiego_a2787	KF799598.1	91	100	49	SRR13165039
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	91	100		
Jan	SB	52	Bacterium ocme08sprp595	JQ657678.1	90	96.55	64	SRR13165038
			Bacterium fme08x95np453l	JQ657514.1	90	96.55		
Jan	SB	70	Uncultured bacterium clone SanDiego_a2787	KF799598.1	90	93.55	66	SRR13165037

			Uncultured bacterium clone SanDiego_a3071	KF799514.1	90	93.55		
Jan	SB	70	Uncultured bacterium clone YE-DC-A15	DQ438329.1	96	96.49	58	SRR13165036
			Uncultured bacterium clone A347_NCI	FJ456660.1	96	94.64		
Feb	SB	45	Bacterium ocme08sprp595	JQ657678.1	82	94.55	64	SRR13165035
			Bacterium fme08x95np453l	JQ657514.1	82	94.55		
Aug	SB	45	Uncultured bacterium clone SanDiego_a2787	KF799598.1	89	97.73	48	SRR13165033
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	89	97.73		
Nov	SB	52	Uncultured bacterium clone SanDiego_a2787	KF799598.1	93	95.56	47	SRR13165032
			Uncultured bacterium clone SanDiego_a3071	KF799514.1	93	95.56		

3.4.8 Phylogenetic analysis

Partial 16S ribosomal RNA sequences of *Crepidula fornicata* positive for Vibrio-like bacteria via CFU presence on TCBS medium, and subsequently via PCR, were aligned, showing some unique sequence types. BLASTn searches revealed consistent close matches (≥90% coverage and identity) for several well-defined *Vibrio* species, including *V. toranzoniae* – a common isolate from healthy bivalves in European waters (see Table 3.3). Phylogenetic trees of amplified nucleotide sequences displayed consistent topology when reconstructed using both maximum likelihood and neighbour-joining routine (Figure 3.16). The tree is comprised of *Vibrio* isolated from diseased and healthy molluscs, as well as environmental isolates (e.g., *V. crassostreae, V. celticus*). A distinct, well supported (99%) grouping of Vibrio-like sequences from both MH and SB sites cluster together, suggesting at least two ecotypes are present in these limpets (green box). The other sequences are distributed among benign and pathogenic bacteria, but they are not closely related to the major invertebrate pathogen, *V. parahaemolyticus.* Due to the small size of the reads (<60 bp), caution is needed when interpreting the potential identity of these bacteria, however, these sequences do represent members of the *Vibrio* genus made-up of at least two ecotypes (perhaps species).

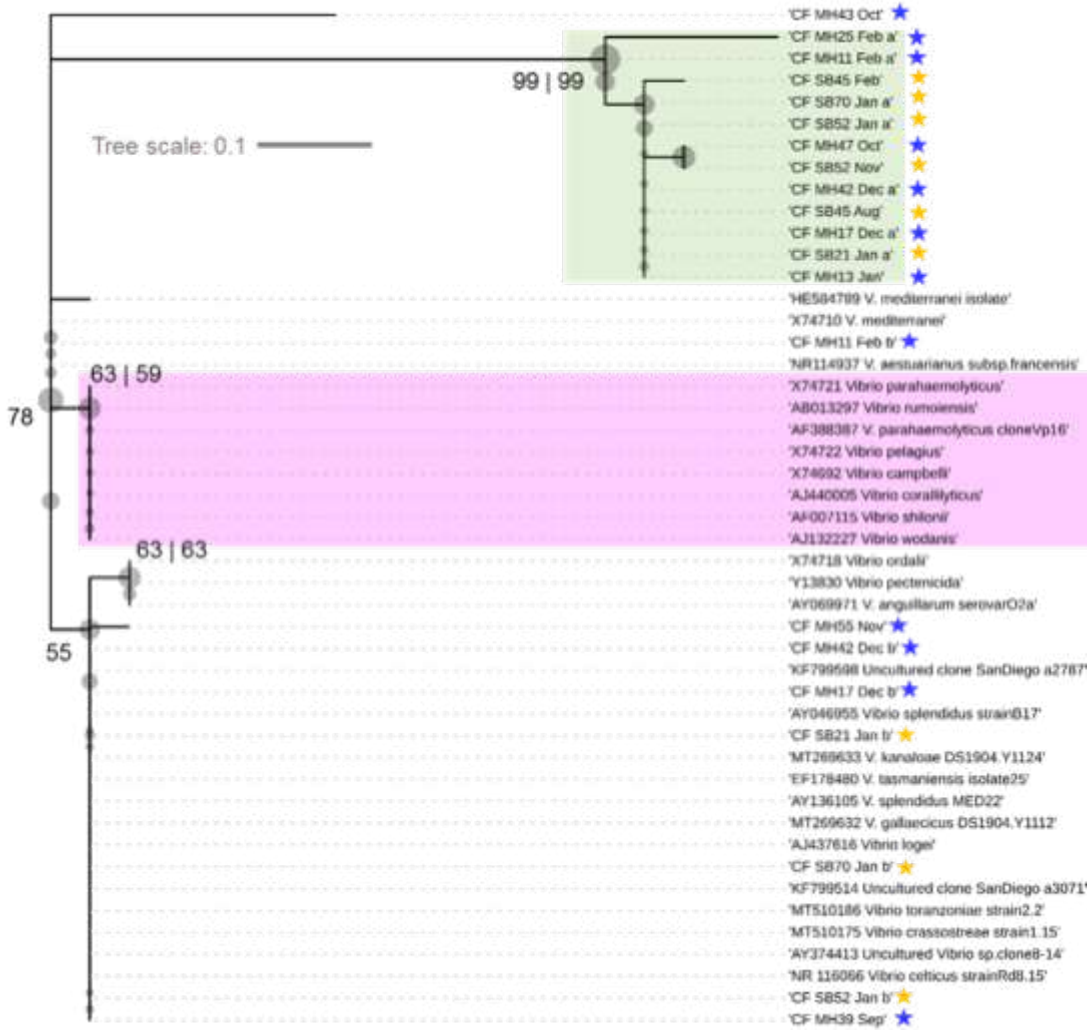

Figure 3.16: Consensus phylogram (unrooted) of the partial 16S rRNA region of sequences recovered from slipper limpets *Crepidula fornicata* (SRR13165025 – SRR13165046). Reference sequences represent the top BLASTn-search results from NCBI plus a broad list of known, geographically distributed *Vibrio* species. Bootstrap support values are depicted as spheres (ML | NJ). The scale represents nucleotide substitutions per site (maximum likelihood (ML) estimation, 1000 bootstrap replicates). All reference sequences used were ≥90% (coverage and identity) matches for the sequences retrieved from limpets. All reads were <60 bp in length. Stars represent sites (blue, MH; yellow, SB). The green box highlights a putative *Vibrio* ecotype. The pink box highlights sequences that cluster with the marine pathogen *V. parahaemolyticus*.

3.4.9 Whole tissue histology of *Crepidula fornicata*

All collected limpets were screened for additional disease-causing agents (e.g., haplosporidians – see Chapter 4), as such, a minimum of 9 individuals per site, per month were processed further for multi-tissue histology: n = 343 (including those limpets with high bacterial loads and with no signs of bacterial presence). Overall, there was no clear evidence of tissue-wide damage or systemic infection that could be attributed to vibriosis. In few individuals, there were signs of haemocyte infiltration, nodule formation, and various states of compromised tissue. Mostly, the gills, the digestive glands and connective tissues appeared normal (Figure 3.17A, Figure 3.18A and Figure 3.19A, respectively) – regardless of the sizeable load of bacteria confirmed via culturing and PCR. One limpet sampled from Milford Haven in May (M40 May), was noticeably compromised across several tissues, albeit still alive. In healthy individuals, the gill filament displayed a continuous, highly ciliated layer and discrete luminal spaces (Figure 3.17A). The gills from the septic animal showed signs of damage to the outer epithelium and many cells in the space around the gills (e.g., lacking cilia), perhaps representing sloughed cells and/or tissue putrefaction (Figure 3.17B). Damage extended to the digestive gland tubules (Figure 3.18B) – haemocytes had clearly infiltrated the tissues, presumably in response to damage (Figure 3.18B), and some tubule architecture appeared dysregulated and displaced. Damage to the digestive gland tubule was also observed in another specimen from Swansea Bay in March (SB4), despite this animal having a low bacterial burden, it was noted that damage did not extend to the rest of the digestive gland (Figure 3.18C). Rod shaped bacteria of various sizes and morphology were visible in the connective tissue (Figure 3.19B), but this may be a late-stage secondary infection where the initial cause – biotic or abiotic – is unclear. Lipofuscin-containing granule abundance and intensity can vary greatly across tissues within an individual, and so it is unclear whether this represents a pathological condition/infection (some links to metal detoxification). The septic limpet (Figure 3.19B) depicts heterogeneous distributions of lipofuscin. Overall, bacterial diseases of this species, based on the two sites surveyed, appear to be very rare with only 1/343 (0.3%) specimen showing significant signs of bacterial disease.

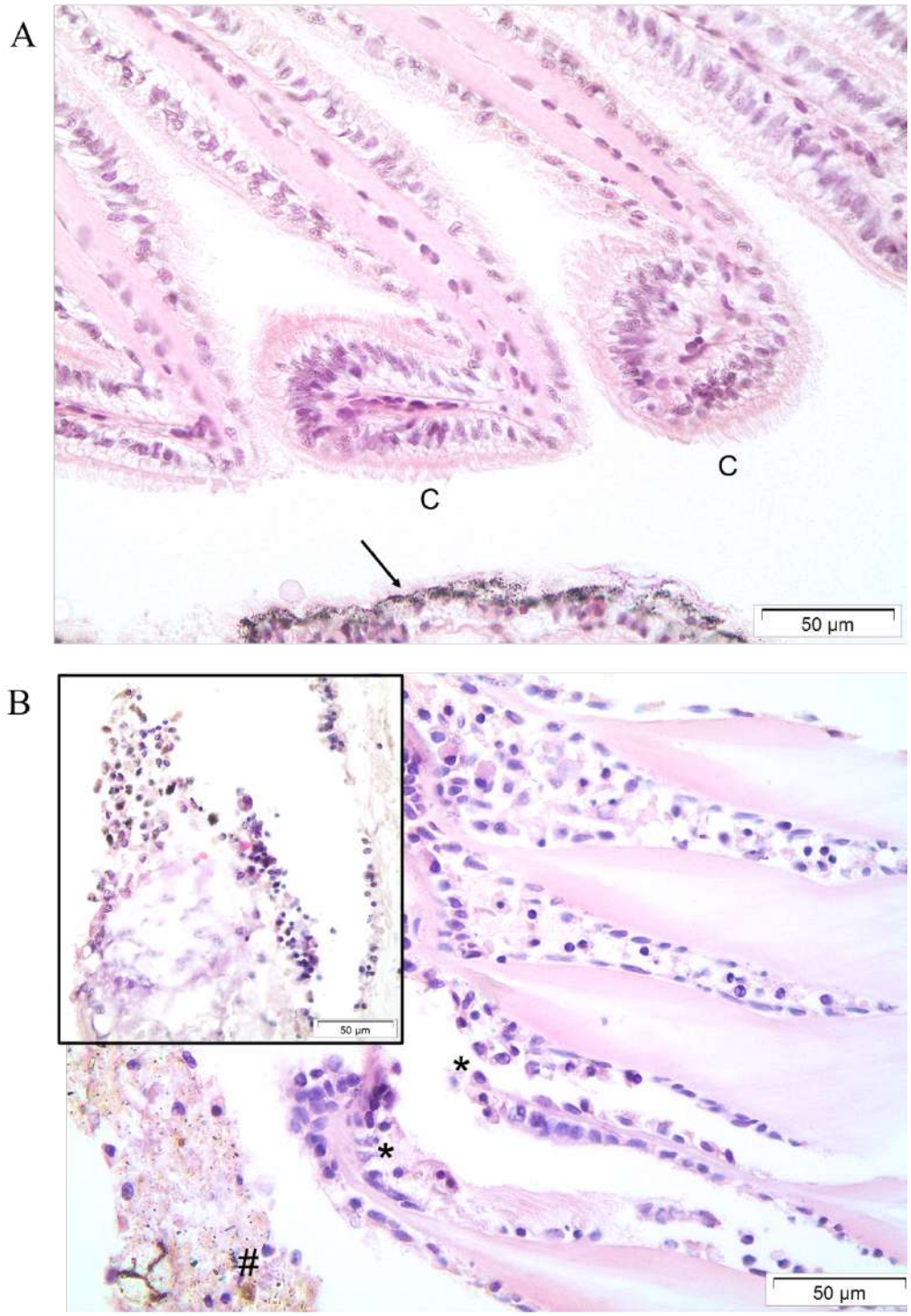

Figure 3.17: Tissue histology of the gill structure of *Crepidula fornicata*. (A) Healthy gill structure, (C) indicates cilia, arrow points to area of melanin deposit. (B) Gill structure from a septic limpet with inset further demonstrating a degradation to the cellular architecture, and lack of distinct cilia that are clearly observed in a healthy gill. Hashtag (#) indicates lipofuscin-like material, asterisk (*) denotes haemocyte presence. L, luminal space

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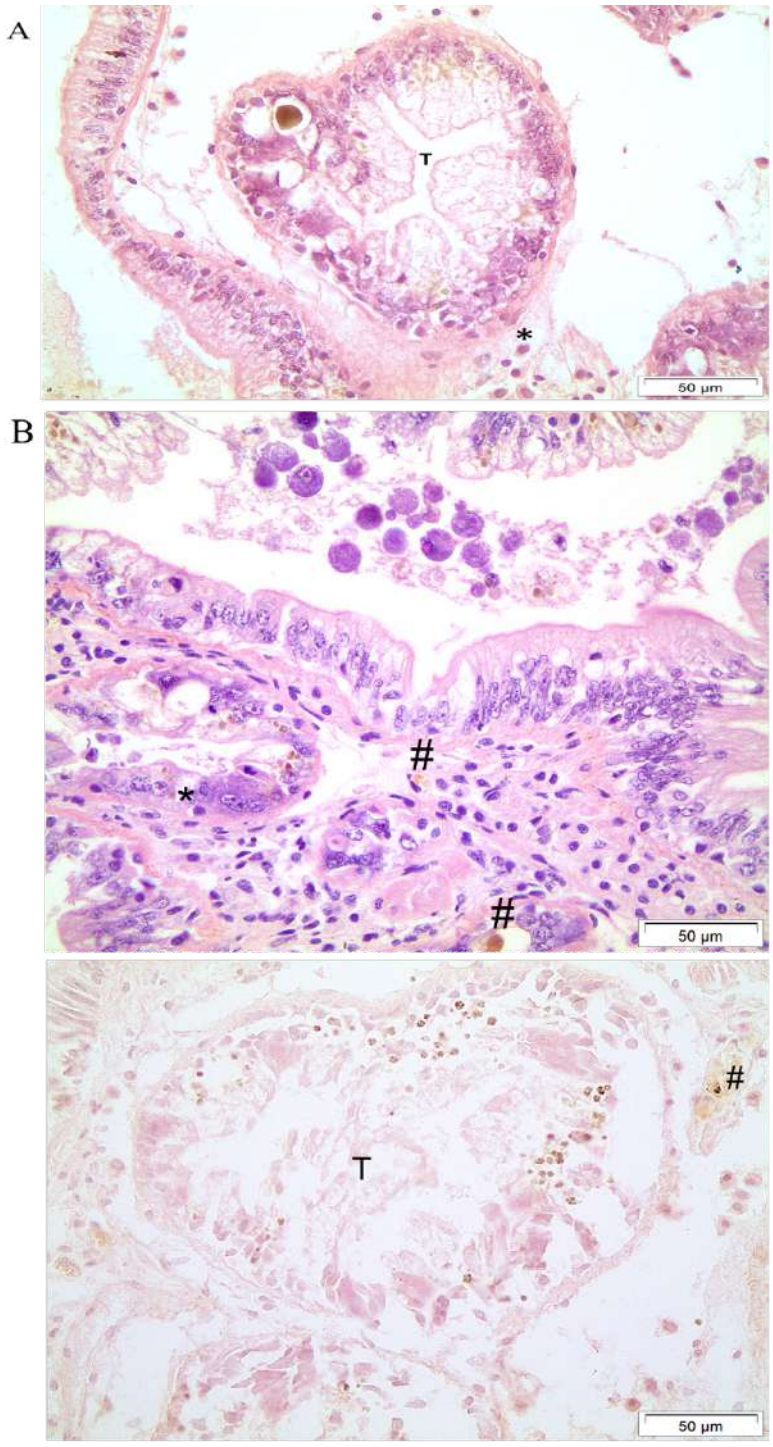

Figure 3.18: Tissue section of the digestive gland of *Crepidula fornicata*. (A) Healthy digestive gland, asterisk denotes haemocyte, T indicates tubule. (B) Digestive gland showing haemocyte infiltration, hashtag (#) indicates lipofuscin-like melanin, asterisk (*) indicates haemocytes. (C) Tubule (T) located in the digestive gland displaying extensive damage, hashtag (#) denotes lipofuscin-like melanin.

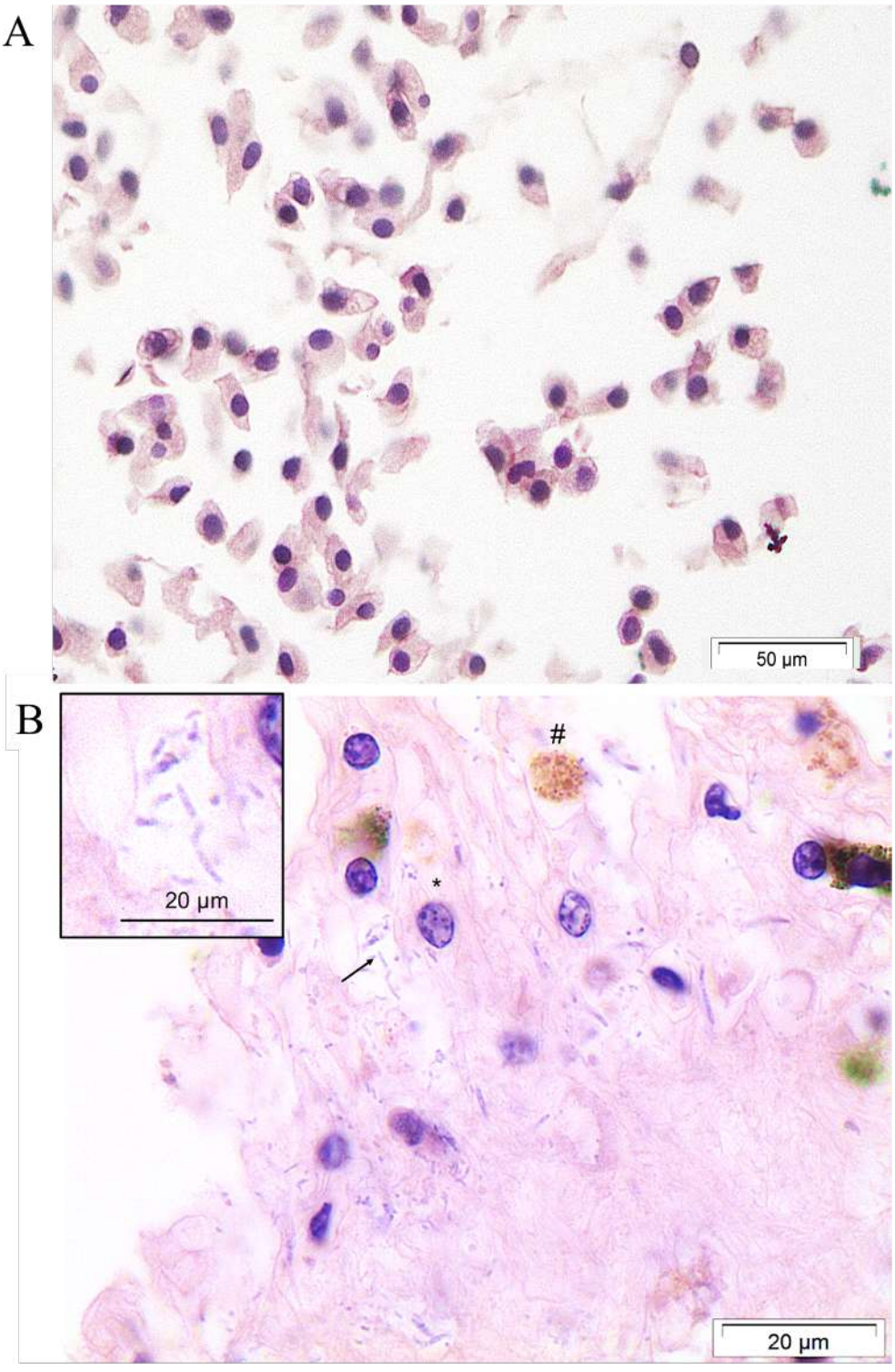

Figure 3.19: Tissue histology of *Crepidula fornicata* connective tissue. (A) Connective tissue without the presence of bacteria, (B) Connective tissue with the presence of rod-shaped bacteria, indicated by arrows, asterisk (*) indicates haemocytes, # denotes area of lipofuscin-like material.

3.5 Discussion

To date, little to no disease or pathogenic agents have been reported in *Crepidula fornicata* other than the presence of the shell boring sponge *Cliona celata* (see Le Cam and Viard, 2011). This chapter aimed to investigate the potential bacterial disease profile of the invasive slipper limpet *C. fornicata,* with a focus on a key aetiological agent in commercial shellfish, namely *Vibrio*. This chapter describes the presence of Vibrio-like bacteria in *C. fornicata* at both sites sampled in south Wales, Swansea Bay, and Milford Haven. Limpets contained a high prevalence of Vibrio-like bacteria detected through PCR, and TCBS agar with over 85% of samples being PCR positive (Figure 3.13). Though a high proportion of the large sample size were PCR positive for Vibrio-like bacteria, there is substantial evidence that these bacteria are not resulting in any disease within the animal. Evidence of a diseased animal would include the visualisation of haemocyte infiltration, nodule formation, and compromised tissue though histology, of which little was noted in only a few individuals. It is possible, that *C. fornicata* simply accumulate bacteria due to their filter feeding nature, thereby acting as a sink for the microbe.

Between sites, limpet populations differed substantially morphometrically. The subtidal population at Swansea Bay were much larger, both in the size of the individual, along with the size of stacks found. Life in the intertidal zone tends to be more stressful for an individual when compared to that of the subtidal zone (Valdivia *et al*., 2011). Individuals found in the intertidal zone, are faced with increased desiccation, osmotic stress, temperature stress, and issues associated with gaseous exchange and metabolic wastes accumulating during the time exposed to air (Rawlings, 1999). Due to their sessile nature, *C. fornicata* found in the intertidal zone may have less opportunity to gather food, than their subtidal counterparts (Diederich *et al*., 2015). These added stressors may help to explain the size differences between those individuals found at Swansea Bay, and Milford Haven. However in a study carried out that focused on the differences in feeding adaptations between intertidal and subtidal *C. fornicata,* Diederich *et al.* (2015) found no significant difference in size between intertidal and

subtidal individuals collected from the same site. The major morphometric difference that was noted in the intertidal and subtidal individuals was that intertidal *C. fornicata* had heavier gills, these larger gills were thought to be a physiological adaptation to reduced feeding times. In other molluscs, a size discrepancy between intertidal and subtidal individuals have been noted, with subtidal animals generally being larger (Sumner, 1981; Gam *et al*., 2008; Tagliarolo *et al*., 2012). Differences in stack sizes may reflect the different sediment types at Swansea Bay and Milford Haven. Bohn *et al*. (2015) found that the average stack size of *C. fornicata* decreased with the increased presence of gravel. They concluded increased availability in gravel resulted in a more dispersed distribution, with newer stacks being more frequently formed. The increased stack sizes in Swansea Bay could be attributable to the bay being relatively protected from wave exposure by Mumbles Head, and sediments mostly consisting of fine to medium sand, and mud (Smith and Shackley, 2006). High densities of *C. fornicata* are commonly associated with areas of finer sediments, as the areas tend to be more sheltered, making a suitable habitat for *C. fornicata* (see de Montaudouin *et al*. 1999). Another potential reason for increased stack size in Swansea Bay is the high proportion of sand, and mud, conspecifics may be forced to settle onto of one another instead of forming new stacks due to a lack of suitable substrate (Bohn *et al*., 2015).

Despite the significant morphometrical differences observed between *Crepidula fornicata* collected at Swansea Bay, and Milford Haven, site was not a significant factor when it came to the probability of an individual testing positive for Vibrio-like bacteria. Instead, seasonality was an important contributing factor. The highest prevalence of Vibrio-like bacteria occurred during summer and late autumn. Beyond season, weight, and the presence of CFU growth on TCBS agar, were key predictors of bacterial presence (Figure 3.15).

The role of seasonality and its relationship with *Vibrio* abundance has been described in numerous studies (Croci *et al*., 2001; Mahmud *et al*., 2008; Di *et al*., 2017; Cruz *et al*., 2020; Yang *et al*., 2021). Water temperatures exceeding 15°C promote rapid growth of vibrios (McLaughlin *et al*., 2005; Baker-Austin *et al*., 2013). The frequency of *Vibrio*

infections has been increasing worldwide, between 1996 and 2006, the average annual incidence increased by 78%, and between 2008 and 2018 the incidence rate increased by 272% (Froelich and Daines, 2020). For example, in the summer of 2015, Canada experienced the highest number of reported cases of human *Vibrio parahaemolyticus* infections ever reported from the consumption of raw oysters (Taylor *et al*., 2018). This outbreak was associated with higher than normal sea surface temperatures (SST > 15 °C), and the incidence rates decreased once SST dropped below 15 °C (Taylor *et al*., 2018). A study carried out by Vezzulli *et al*. (2012) demonstrated that during the last 50 years, *Vibrio* species such as *V. cholera* increased in dominance within plankton associated bacterial communities of the North Sea, coinciding with an unprecedented rise in *Vibrio* related bathing infections. They were able to show that an increase in sea surface temperature explained 45% of the variance in *Vibrio* data. Through the use of archived plankton samples collected between 1958 and 2011, Vezzulli *et al*. (2016) were able to demonstrate that in nine areas of the North Atlantic and North Sea a correlation between climate and changes in *Vibrio* abundance. At the Irish Sea site, the *Vibrio* index which measures the proportion of *Vibrio* bacteria in relation to total bacteria was measured at -1.3 ± 0.2 from 1990-1999, and increased to 1.0 ± 0.3 during the period 2000-2011 (Vezzulli *et al*., 2016). This chapter found that CFU growth on the *Vibrio* specific medium TCBS followed a similar pattern observed in *Vibrio* PCR positive samples. CFU growth was highest in Summer, and lowest in Winter (Figure 3.8). A large number of studies use TCBS agar as a selective medium for the growth of *Vibrio* spp. from the marine environment (Donovan and van Netten, 1995; Cavallo and Stabili, 2002; Gyraite *et al*., 2019). This chapter found that growth of bacteria on TCBS medium was a useful indicator for a limpet testing positive for Vibrio-like bacteria through the use of PCR.

Marine filter feeders, can accumulate high numbers of bacteria from their surrounding environment (Ciacci *et al*., 2009). Molluscs can be found with levels of *Vibrio* in their tissue of 10^5 CFU/g or higher, this being up to 100-fold higher than that of the surround water (Froelich *et al*., 2017). Due to the stacking nature of *C. fornicata*, whereby younger, smaller, individuals pile on top of older, larger individuals (Collin,

1995), the larger animals will also be the oldest. Larger/older filter feeders appear to have an increased exposure to bacteria, as well as a number of other pathogens such as Haplosporidia, and trematodes due to an increase in filtration capacity and potentially an increased exposure over time (Albuixech-Martí *et al*., 2020; Austin *et al*., 2019; Boehs *et al*., 2010; Lasiak, 1993; Martinez and de Oliveira, 2010).

Haemocytes in molluscs are found in the circulating haemolymph and are involved in a range of biological processes. This chapter found that circulating haemocytes in the haemolymph of *C. fornicata* varied seasonally, regardless of sample location (Figure 3.9). Seasonal variation in haemocytes has also been described in other molluscs (Voltey *et al*., 1999; Barth *et al*., 2005; Suljević *et al*., 2018). In lab experiments carried out to examine the effect of elevated temperatures on the haemocyte response of the clam *Chamelea gallina*, Monari *et al.* (2007) showed that at the highest temperature of 30 °C a significant increase in haemocyte counts occurred. Though in this study, haemocyte numbers followed a similar pattern to that of the presence of Vibrio-like bacteria, it needs to be taken into account that haemocyte numbers can vary for a number of reasons, besides the presence of bacteria, e.g., reproductive cycle, Santarem *et al*. (1994) found haemocyte numbers in *Mytilus galloprovincialis* to be at their lowest post-spawning. Variation can also result as a response to environmental changes. Cheng *et al*. (2004) found that in abalone *Haliotis diversicolor supertexta* exposed to higher ammonia levels, haemocyte counts decreased. Environmental contaminants that find their way into water sources have also been seen to impact haemocyte numbers. Lorenzon *et al*. (2001) studied the negative effects of heavy metal exposure on circulating haemocyte numbers in the shrimp *Palaemon elegans;* and found a decrease in circulating haemocyte counts in the first 8 h following exposure, with levels returning to base line over the subsequent 16 h.

Through the use of direct Sanger sequencing, a number of *Vibrio* species present in *C. fornicata* were identified. Notably these matches included species that are described in literature as potentially pathogenic, though they did not appear to be pathogenic to *C.*

fornicata. These matches include the likes of *Vibrio mediterranei, V. tasmaniensis, V. crassostreae*, and *V. celticus*, all of which have been demonstrated to have negative effects on oysters, mussels, and clams (Tall *et al*., 2013; Romalde *et al*., 2014; Vanhove *et al*., 2015; Dubert *et al*., 2017; Andree *et al*., 2020). It is important to note, that the generated sequences from the *Vibrio* specific PCR undertaken in this study were of a small fragment size. As a result of the sequences being short, it is possible for them to match closely with a high proportion of the *Vibrio* sequences held within the NCBI nucleotide database. Despite the majority of *C. fornicata* testing positive through PCR for Vibrio-like bacteria, there is limited evidence of these individuals being negatively affected by their high bacterial load. It would appear that *C. fornicata at* the chosen study sites are resilient to the presence of large bacterial burdens. Tissue screening using histology revealed most limpets as 'pristine', with very few signs of tissue damage or bacterial infiltration of solid tissues. A single limpet appeared moribund due to the very high bacterial load, and signs of gross proliferation among tissues, necrosis and potential putrefaction. In a study carried out by Le Cam and Viard (2011), that recorded the presence of the shell boring sponge *Cliona celata,* it was observed that despite *C. fornicata* suffering from high parasite loads caused by *Cliona celata*, they suffered limited side-effects, i.e., no mention of secondary infections. Moreover, other commercially important species in the same area developed additional damage linked to the presence of the parasitic sponge.

3.5 Conclusions

The results from this chapter help to provide a better understanding of the disease status of the invasive slipper limpet *C. fornicata,* along with some of the potential variables that influence the presence of Vibrio-like bacteria. Overall *C. fornicata* had high levels of Vibrio-like bacteria. Observations were made across multiple tissues via histology, and a distinct lack of disease signatures were noted e.g., tissue damage, or immune cell reactivity such as haemocyte infiltration, except for one individual suffering from systemic bacteriosis. The patterns observed with the presence of Vibrio-like bacteria appear to be independent of location and sex, and instead any variation

observed is linked with seasonality and limpet size. Importantly, there is no substantial evidence that would suggest *C. fornicata* are acting as reservoirs for any notifiable pathogens of shellfish and/or humans. However, it is possible that *C. fornicata* are acting as sinks for Vibrio-like bacteria, likely due to their general ecology, i.e., being a filter feeding mollusc.

Chapter 4 Symbionts and pathobionts of *Crepidula fornicata*

4.1 Abstract

Two populations of the invasive slipper limpet *Crepidula fornicata* were sampled in Wales, U.K., to determine the presence/absence of important pathogens known to affect commercially important shellfish species. Milford Haven represents an important area for commercial activity, and Swansea Bay is a restoration site for native oyster. A multi-resource screen, including PCR and histological approaches were used to assess 1,800 individuals over 12 months for haplosporidians, microsporidians, and paramyxids. Although PCR-based detection indicated the presence of disease-causing agents in limpets, there were no signs of infection when assessed histologically, or when PCR amplicons were sequenced. Whole tissue histology did reveal the presence of trematodes, turbellarians, and an apicomplexan-like parasite in the epithelial cells of the alimentary canal. In total 57/343 *C. fornicata* screen via histology were identified as to be carrying a turbellarian, and 59/343 were observed to contain an apicomplexan-like parasite. A seasonal effect was observed with regards to infection with turbellarians (57/343) and apicomplexan-like parasites (59/343). The winter months had the highest prevalence of apicomplexan-like parasites while turbellarians were most prevalent during the summer months. Turbellarian prevalence was greater at Milford Haven than in Swansea Bay. A small proportion of female *C. fornicata* were seen to have gonadal tissue infected with trematode-like parasites in both Swansea Bay and Milford Haven during the summer months that resulted in parasitic castration/sterilisation. Overall, these data suggest that *C. fornicata* are not susceptible to substantive parasitic infections outside of their native range, which may contribute in part to their invasion success.

4.2 Introduction

Invasive alien species can pose a significant threat to native biodiversity and alter ecosystem functioning in marine and freshwater environments (Sala *et al.*, 2000). According to the EU's Invasive Alien Species (IAS) regulations, an invasive alien species is any species found outside of its natural range and whose introduction and/or spread has been deemed to threaten or have adverse impacts upon biodiversity and related ecosystems (European Union, 2014). For a quarter of IAS listed on the International Union for Conservation of Nature (IUCN), "100 of the world's worst" environmental impacts list are linked to diseases in wildlife (Hatcher *et al.*, 2012). IAS can be a significant source of "pathogen pollution", which is defined as the human-mediated introduction of a pathogen to a new host or region, often unintentionally (Roy *et al.*, 2017). IAS that act as vectors for infectious agents can cause parasite "spillover" into native populations, causing an emerging disease (Costello *et al.*, 2021). When an invasive species acts as an alternative host for native or already established parasites, they may result in the parasite population size rising, leading to increased levels of infection in native host populations, i.e. "parasite spillback" (Kelly *et al.*, 2009). IAS also have the potential to dilute the native parasite population and thus reduce the disease risk for native species, termed "Transmission interference" or "Parasite dilution" (Thieltges *et al.*, 2009; Poulin *et al.*, 2011; Goedknegt *et al.*, 2016). On the other hand, maintaining or developing parasitic infections can lead to regulation for invasive populations, possibly controlling population size and reducing the impact of the invasive through impacting host fitness (Prenter *et al.*, 2004; Tompkins *et al.*, 2011; Dunn *et al.*, 2012). Invasive species generally have fewer numbers and a less diverse range of parasites than their counterparts in their native ranges (Blakeslee *et al.*, 2013). "Parasite escapes" occurs due to the loss of native-range parasites and from not attaining an equivalent amount of new parasites in its invaded area (Torchin *et al.*, 2003).

The definition of what constitutes a parasite varies depending on the field of study. In general, parasitism can be defined as the close association of two organisms, in which one (the parasite), during at least one life-history stage, depends on the other (the host) and directly gains some nourishment and/or shelter but does not intentionally kill the

host (Morris and Costello, 2019). The term parasite often includes macroparasites, i.e. those in which direct multiplication in the definitive host is low or completely absent (e.g. digenean trematodes) and microparasites (e.g. protozoa, bacteria, and viruses) that show high rates of direct reproduction within the host (Anderson and May, 1979). Both macro- and micro-parasites can affect both wild aquatic species and commercial species used in cultured systems and local, often traditional, shellfisheries (Costello *et al*., 2021). Examples of microparasites that have been known to infect shellfish include haplosporidians, microsporidians and paramyxids (Azevedo *et al*., 2008; Bhaby, 2015; Carrasco *et al*., 2015; Kristmundsson *et al*., 2015; Stentiford *et al*., 2016; Lynch *et al*., 2019). Haplosporidians (Figure 4.1) are protozoan parasites that are considered significant pathogens of concern for aquatic animal health managers and shellfish industries, resulting in some of the most devasting marine disease events on record (Arzul and Carnegie, 2015). An outbreak of *Haplosporidium nelsoni* along the mid- Atlantic coast of the USA resulted in eastern oyster (*Crassostrea virginica*) mortalities of over 90% (Burreson *et al*., 2000). High mortalities of up to 90% in the blackfoot abalone *Haliotis iris* as the result of a haplosporidian outbreak that occurred at a New Zealand commercial culture facility were reported in April 2000 (Diggles *et al*., 2002).

Protozoan paramyxids (Figure 4.1) such as *Marteilia* spp. have seriously impacted cultured stocks of the flat oyster *Ostrea edulis,* European mussels *Mytilus edulis* and *M. galloprovincialis*, and European cockles *Cerastoderma edule* (see Fuentes *et al*. 2002; Berthe *et al*. 2004; Carrasco *et al*. 2012; Pękala and Paździor 2012). Since the 1970s, *Marteilia sydneyi* has caused outbreaks of QX disease in the Sydney rock oyster, *Saccostrea glomerata* (Rubio *et al*., 2013).

Microsporidians (Figure 4.2) are a broad group of unicellular obligate intracellular spore-forming parasites (Han *et al*., 2020). The microsporidian parasite *Steinhausia mytilovum* has been observed parasitising the oocytes of the Mediterranean mussel *Mytilus galloprovincialis* (Sagristà *et al*., 1998; Rayyan and Chintiroglou, 2003).

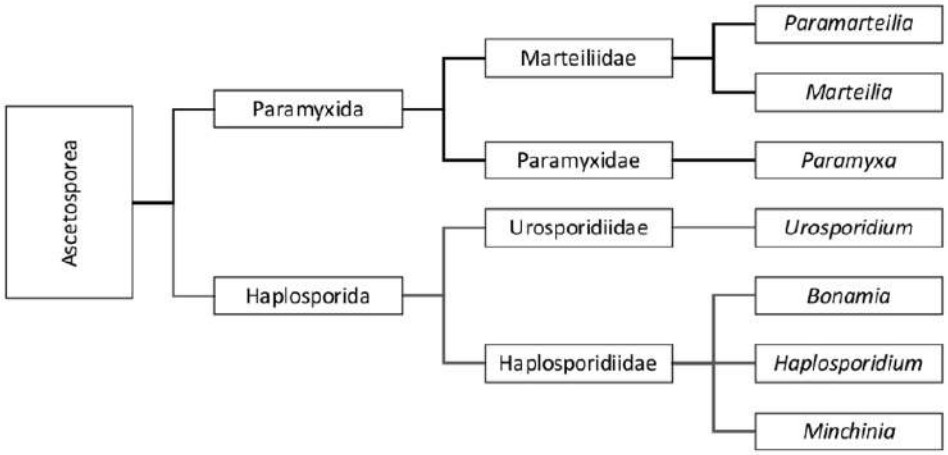

Figure 4.1: Taxonomic description of haplosporidians, and paramyxids, belonging to the Class Ascetosporea.

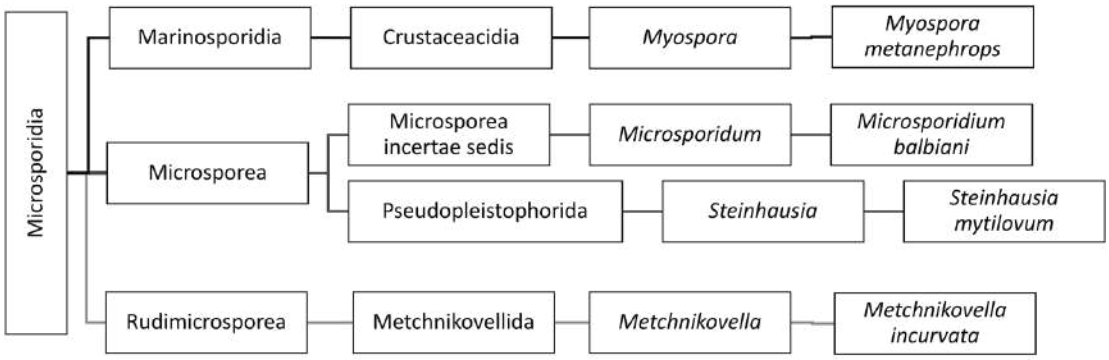

Figure 4.2: Taxonomic description of the class Microsporidia. There are approximately 200 genera and 1,400 formally described species. This diagram gives an example of 4 species.

One invasive alien species that current knowledge of its pathogen profile is lacking is the slipper limpet *Crepidula fornicata. Crepidula fornicata is* now a frequently observed IAS, especially in areas of the U.K., such as Wales, and across Europe in countries such as France (Fitzgerald, 2007; Bohn *et al*., 2015). *Crepidula fornicata* was placed on the "Non-native species subject to restrictions under Regulations 49 and 50" list in the Third Schedule of the European Communities (Birds and Natural Habitats) Regulations 2011, S1477/2011, and was subsequently designated as a "high risk" invasive species in Ireland and Northern Ireland, in 2012. In the U.K., *C. fornicata* is listed under Schedule 9 of the Wildlife and Countryside Act 1981. The UK Marine Management Organisation

(MMO) deemed the release of live or fresh slipper limpets into the wild to be an offence in 2015 in hopes to minimise any further spread (MMO, 2015).

This chapter aimed to perform a large-scale temporal and spatial survey for the presence of haplosporidians, microsporidians and paramyxids in the invasive non- native slipper limpet *C. fornicata across* two sites in Wales, U.K (Swansea Bay and Milford Haven). To ascertain the presence of these parasites, 75 samples per site were collected monthly from January 2019 – December 2019 and screened using PCR (haemolymph) and examined histologically (solid tissues).

4.3 Materials and methods

4.3.1 Study Sites

Sampling for both intertidal and subtidal live *C. fornicata* (n = 1,800) took place at two sites in south Wales that have well-established populations. Subtidal collection took place at Swansea Bay (51.570345, -3.974591; n = 900), and intertidal collection took place at Milford Haven (n = 900; 51.7042, -4.971295). Details of these sample sites are given in Chapter 3.1.1

4.3.2 Laboratory regime

The laboratory regime as described in Chapter 3.3.2 was followed to process the *Crepidula fornicata* for this study. Briefly, morphometric measurements were collected for each individual (wet weight (g), length (mm), width (mm), and position in the stack. The sex of the specimen was determined and assigned as male (presence of developed penis), female (absence of penis), or transitionary (reduced penis). Haemolymph was aseptically collected from each specimen by first removing the flesh from the shell and allowing the haemolymph to pool in the cavity. A sterile 1 mL syringe with a 22G needle attached was used to aspirate the haemolymph.

4.3.3 Tissue histology

Whole tissue histology was carried out to screen a subset of animals (n = 343) to visualise any potential pathogens and immune responses. Animals were chosen for histology based on an initial positive PCR outcome for the disease screen targets. A minimum of 9 samples per month per site were selected. The process has been described in detail in Chapter 2.3.8.

4.3.4 Molecular diagnostic techniques

DNA extraction

DNA extraction was carried out as described in Chapter 3.3.4, using 100 µL of *C. fornicata* haemolymph using a Sigma Aldrich GenElute ™ Blood Genomic DNA Kit. It was not possible to extract a sufficient quantity of haemolymph from 29/1,800 specimens. In such cases, 20 mg of solid foot tissue was used.

Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in 25 µL total reaction volumes using 2x Master Mix (New England Biolabs, oligonucleotide primers synthesised by Eurofins (Ebersberg, Germany), 1 µL DNA, and performed using a PCR thermocycler (BioRad Laboratories Inc., Hemel Hempstead, UK.). PCR products were visualised on a 2% agarose/TBE gel with Greensafe premium nucleic acid stain (NZYTech, Portugal). PCR conditions for each target species are as described in Table 4.1.

Table 4.1: Forward and reverse primer sequences used for the amplification of haplosporidians, microsporidians, and paramyxids. Thermocyling conditions are also described

Target pathogen	Primers				Thermocycler settings			Amplicon size (bp)	References
	Dir.	Name	Sequence (5'–3')	Final conc (µM)	Temp (°C)	Time	No. of cycles		
***Haplosporidia* sp. round 1**	Fwd	C5fHap	GTAGTCCCARCYATAACBATGTC	1	95	5 min	30	N.A.[#]	(Hartikainen *et al*., 2014)
	Rev	Sb1n	GATCCHTCYGCAGGTTCACCTACG		95	30 s			
					65	45 s			
***Haplosporidia* sp. round 2**	Fwd	V5fHapl	GGACTCRGGGGGAAGTATGCT	1	95	5 min	30	650	(Hartikainen *et al*., 2014)
	Rev	Sb2nHap	CCTTGTTACGACTTBTYCTTCCTC		95	30 s			
					65	45 s			
					72	1 min			
					72	10 min			
Microsporidians	Fwd	CTMicrosp-G	CACCAGGTTGATTCTGCCTGAC	0.5	94	5 min	35	1100-1300	(Fedorko *et al*., 1995; Stentiford *et al*., 2018)
	Rev	Microsp1342r	ACGGGCGGTGTGTACAAAGAACAG		94	30 s			
					63	30 s			
					72	90 s			
					72	10 min			
Paramyxids (round 1)	Fwd	Para1+fN	GCGAGGGGTAAAATCTGAT	1	95	3 min	42	N.A.[#]	(Ward *et al*., 2016)
	Rev	ParaGENrDBn	GTGTACAAAGGACAGGGACT		95	30 s			
					67	1 min			
					72	1 min			
					72	5 min			
Paramyxids (round 2)	Fwd	Para3+fN	GGCTTCTGGGAGATTACGG	1	95	3 min	42	450	(Ward *et al*., 2016)
	Rev	Para2+rN	TCGATCCCRACTGRGCC		95	30 s			
					62	1 min			
					72	1 min			
					72	5 min			

Direct sanger sequencing

Amplicons obtained through PCR were purified using HT ExoSAP-IT™ Fast high-throughput PCR product clean-up (Thermo Fisher Scientific, Altrincham, U.K.). Sanger sequencing was carried out by Eurofins. The generated sequences were trimmed manually of primer regions and searched against the National Centre for Biotechnology Information (NCBI) nucleotide database using BLASTn (Basic Local Alignment Search Tool). Query Coverage (Q.C.), Maximum Identity (MI) and E-value data were recorded.

4.3.5 Statistical analyses

The sample size for carrying out the disease screen was calculated as described in Chapter 3.3.5. Binomial Logistical Regression modelling was carried out to determine if any specific variables contributed to the presence of any of the target pathogens, i.e., haplosporidians, paramyxids, and microsporidians. This was carried out using the Logit link functions found in the MASS library (following Bernoulli distributions). An information-theoretical approach was utilised for both model selection and assessment of model performance (Richards, 2005). A detailed explanation of the process used to reduce the full model is described in Chapter 3.3.5. All other statistical tests (tests of normality, Kruskal-Wallis, Mann-Whitney, Chi-square) were produced using GraphPad Prism v.9 for Mac (GraphPad Software, La Jolla California USA). Figures were prepared in GraphPad PRISM v9.

4.4 Results

4.4.1 Molecular screening

Overall, 1,800 *Crepidula fornicata* collected over 12 months, 900 from Milford Haven and 900 from Swansea Bay, were screened for the presence of haplosporidians, paramyxids, and microsporidians using a PCR based method. Of these 1800, 14% (257/1800), were PCR positive for haplosporidians, 1.5% (27/1800), for paramyxids, and 0.5% (10/1800) for microsporidians.

In total, 257/1800 *Crepidula fornicata* tested positive through PCR for the presence of haplosporidians – with amplicons of the expected size. A significant between site difference occurred (X^2_1 (n=1800) =13.73., P = 0.0002; Chi-square;Figure 4.3A), with 17.3% (156/900) of limpets testing positive from Milford Haven, and 11.2 % (101/900) collected at Swansea Bay being PCR positive for haplosporidians. Season appeared to affect the number of haplosporidian PCR positive samples that were observed (P = 0.04; Kruskal Wallis; Figure 4.3B). Autumn had the highest percentage of PCR positive samples with 24% (106/450) of individuals being PCR-positive for haplosporidians, and spring having the lowest prevalence with only 5% of individuals collected during this period being PCR-positive (22/450). A strong correlation between month and the number of PCR positive individuals was observed (P = 0.004; Figure 4.3C). November had the highest prevalence, with 26% of sampled individuals being PCR-positive (40/150), and February with the lowest with zero positive samples. Female *Crepidula fornicata* were more likely to be PCR-positive for haplosporidians when compared to that of male or transitional limpets (X^2_2(n=1800)=52.13, P < 0.0001; Chi-square). The size of the individual did not influence whether they were PCR positive for haplosporidians (P = 0.393; Mann-Whitney; Figure 4.3E). When the results were further looked at, at a site-specific level, some differences compared to the pooled data were observed. At Milford Haven, the prevalence was at its highest in autumn, whereas summer at Swansea Bay had the highest prevalence of PCR positive *Crepidula fornicata* (Figure 4.3F). October at Milford Haven was the highest month of PCR positive samples (37%, 29/75), and at Swansea Bay, numbers peaked during June (24%, 18/75).

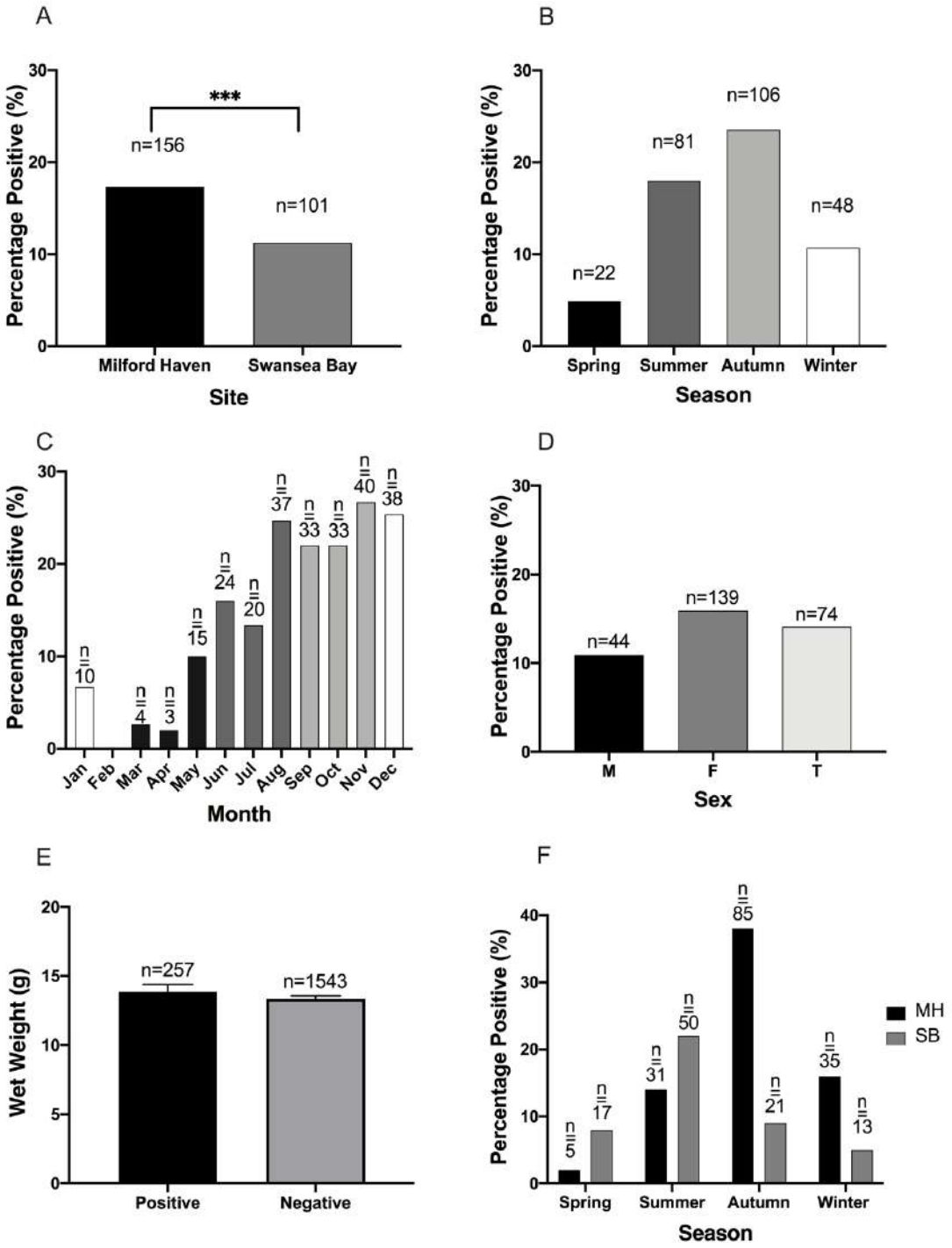

Figure 4.3: Site influence on the rate of haplosporidian PCR-positive *Crepidula fornicata* collected from Milford Haven (n=900) and Swansea Bay (n=900). (B) Seasonal variation in the presence of haplosporidian PCR-positive individuals sampled across the four seasons (n=450/Season). (C) Monthly changes in the number of PCR-positive individuals sampled from Jan-Dec 19 (n=150/month). Percentage prevalence of haplosporidian PCR-positive across the different sexes i.e. Male (M; n=403), Female (F; n=873), and Transitionary (T; n=524) *C. fornicata*. (E) Changes in the Haplosporidia percentage positive (%) PCR-positive rate as a result of the wet weight (g) of the individual (n=1800). (F) Between site variation in the percentage of haplosporidian PCR-positive *C. fornicata* during the four seasons across Spring (n=225), Summer (n=225), Autumn (n=225), and Winter (n=225) per site at Milford Haven and Swansea Bay).

In total, 27 (out of 1,800) samples were PCR positive for the presence of paramyxids. No significant differences were observed between Milford Haven and Swansea Bay with regards to the numbers of PCR positive individuals (X^2_1(n=1800)=.3384, P=0.5607; Chi-square; Figure 4.4A). Milford Haven had 12/900 positive individuals, and Swansea Bay had 15/900 positive *C. fornicata*. No significant seasonal variation was observed (X^2_3, (n=1800)=4.023, P =0.2590; Chi-square; Figure 4.4B). January had the highest number of PCR positive samples (6/150), with many months having zero cases (Feb, Mar, May, Jun, Sep; Figure 4.4C). Due to the overall small number of PCR positive individuals, it was not possible to determine the significance of the monthly variation statistically. No significant difference was observed in the rate of PCR positive Male, Female, and/or Transitionary *C. fornicata* (X^2_2 (n=1800) =.0043, P=0.9978; Chi-square); Figure 4.4D). The size of the individual did not appear to influence the outcome of the PCR test either (P = 0.989; Mann-Whitney; Figure 4.4E). Samples collected during the summer months at Milford Haven had the highest rates of PCR-positive individuals, whereas those collected during winter at Swansea Bay had the highest rates (Figure 4.4F).

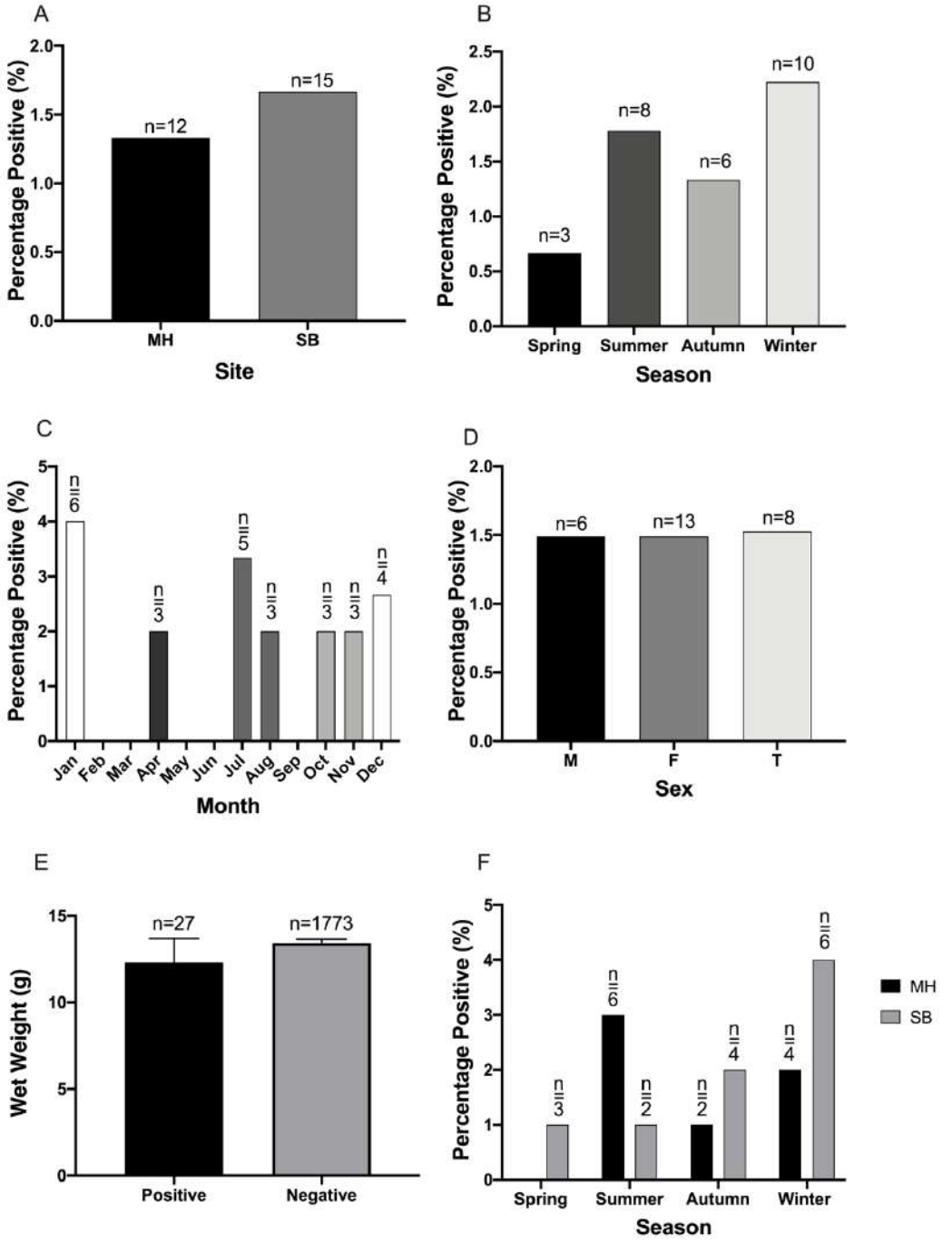

Figure 4.4: Site influence on the rate of paramyxid PCR-positive *Crepidula fornicata* collected from Milford Haven (n=900) and Swansea Bay (n=900). B) Seasonal variation in the presence of paramyxid PCR-positive individuals sampled across the four seasons (n=450/Season). (C) Monthly changes in the number of PCR-positive individuals sampled from Jan-Dec 19 (n=150/month). (D) Percentage prevalence of paramyxid PCR-positive individuals across the different sexes i.e., Male (M; n=403), Female (F; n=873), and Transitionary (T; n=524) *C. fornicata.* (E) Changes in the percentage positive (%) paramyxid PCR-positive rate as a result of the wet weight (g) of the individual (n=1800). (F) Between site variation in the percentage of paramyxid PCR-positive *C. fornicata* during the four seasons across Spring (n=225), Summer (n=225), Autumn (n=225), and Winter (n=225) at Milford Haven and Swansea Bay).

Only 10/1800 samples were PCR positive for the presence of Microsporidia. Of those limpets, 8 originated from Swansea Bay and two from Milford Haven (Figure 4.5A). Though a difference in the number of positive individuals was observed, this was not statistically significant (P = 0.1084; Fishers Exact Test). Autumn had the highest proportion of PCR-positive samples (9/450), Summer had only one positive sample throughout the season. Spring and winter months had no PCR-positive individuals (Figure 4.5B). October and November had the highest number of PCR positive samples with four and three, respectively (Figure 4.5C). Though seasonal and monthly variation was observed, it was not possible to test this statistically due to the small number of positive individuals. Some variation in the number of PCR positive Male (2/10), Female (4/10), and Transitionary (4/10) individuals were observed (Figure 4.5D). Size had an impact on the rate of Microsporidia PCR-positive individuals, with larger (n=10; 21.91 g ± 3.35g, mean ± S.E.) individuals having a higher prevalence than those smaller individuals (n=1790; 13.38 g ± 0.2033 g, mean ± S.E.; P = 0.005; Mann-Whitney; Figure 4.5E). Autumn at Swansea Bay was the only season where PCR-positive individuals were found. At Milford Haven, PCR-positive samples were collected in spring and summer (Figure 4.5F).

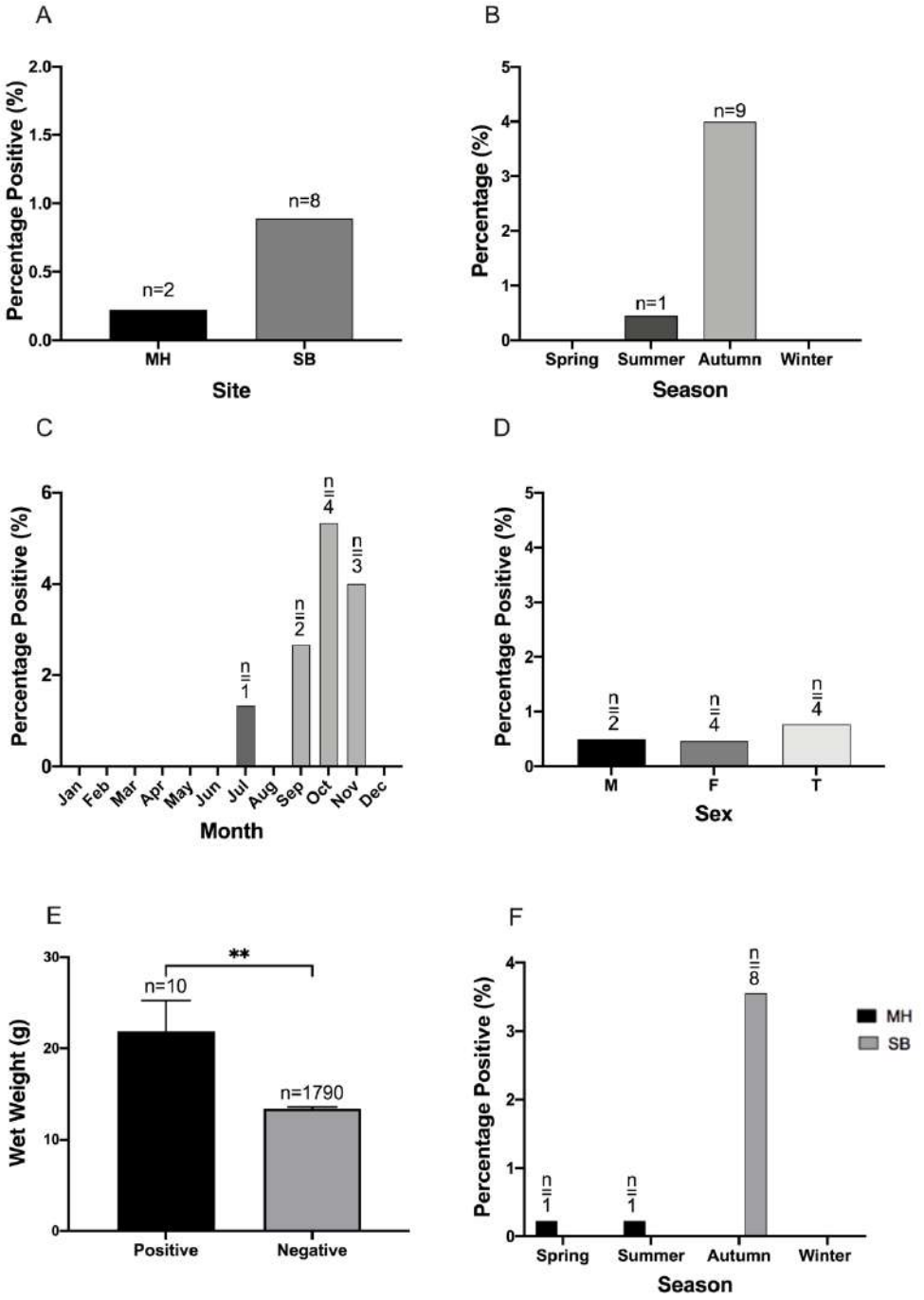

Figure 4.5: (A) Site influence on the rate of microsporidian PCR-positive *Crepidula fornicata* collected from Milford Haven (n=900) and Swansea Bay (n=900). (B) Seasonal variation in the presence of microsporidian PCR-positive individuals sampled across the four seasons (n=450/Season). (C) Monthly changes in the number of PCR-positive individuals sampled from Jan-Dec 19 (n=150/month). (D) Percentage prevalence of microsporidian PCR-positive across the different sexes i.e. Male (M; n=403), Female (F; n=873), and Transitionary (T; n=524) *C. fornicata.* (E) Changes in the percentage positive (%) variation in microsporidian PCR-positive rate as a result of the wet weight (g) of the individual (n=1800). (F) Between site variation in the percentage of microsporidian PCR-positive *C. fornicata during* the four seasons across Spring (n=225), Summer (n=225), Autumn (n=225), and Winter (n=225) at Milford Haven and Swansea Bay).

4.4.2 Factors associated with the presence of haplosporidians, paramyxids, and microsporidians

Biometric data recorded from *Crepidula fornicata,* including size, sex, and position in stack, along with collection site, were included in the initial Binomial Logistic Regression (BLR) model(s) to determine if any variables contributed significantly to *Crepidula fornicata* being PCR-positive for the presence of haplosporidians, paramyxids or microsporidians in the samples collected from either location.

Haplosporidians

The main contributing variables to *Crepidula fornicata* individuals being PCR-positive for the presence of haplosporidians was determined to be Site, Season and Size (Table 4.2) Swansea Bay samples were found to have significantly fewer PCR- positive individuals ($P< 0.0001$). Autumn was the season with the highest number of haplosporidian PCR positive individuals, with spring, summer, and winter having significantly fewer cases ($P<0.001$, $P=0.035$, $P<0.0001$, respectively). Larger *Crepidula fornicata* were found to be more likely to be PCR-positive for haplosporidians ($P = 0.000832$).

Paramyxids

The BLR model revealed that there were no variables significantly contributing to the rate of paramyxid PCR-positive *Crepidula fornicata* sampled from Milford Haven and Swansea Bay (Table 4.2) Though there was some variability in the rates of PCR- positive individuals between site, this was not statistically significant ($P = 0.220$). A subtle seasonal effect was observed, with winter having the highest count of paramyxid PCR positive samples, but again this was not statistically significant ($P = 0.34402$). Females were more likely to be PCR-positive when compared to Males and Transitionary, but not significantly so ($P = 0.513$, $P = 0.854$, respectively).

Microsporidians

The final model determined that wet weight was a main contributing factor to a *Crepidula fornicata* individual being PCR positive, i.e., larger limpets ($P = 0.000341$; Table 4.2). Atemporal effect was apparent, with Autumn having the highest rate of PCR

positive individuals when compared to Spring (P =0.003). Spring and Winter did not have any detectable microsporidian signals.

Table 4.2: Binomial logistical regression model testing the influence of biometric and environmental predictor variables on the presence of haplosporidian, paramyxid, and microsporidian in *Crepidula fornicata* sampled from Swansea Bay and Milford Haven from Jan 19 – Dec 19

Model	**Predictor Variable**	**Estimate Slope**	**S.E.**	***P*-value**
Haplosporidian	SiteSB	-1.01611	0.21143	**< 0.0001**
Reduced Model				
	SeasonSpring	-1.77393	0.24871	**< 0.0001**
	SeasonSummer	-0.35504	0.16852	0.035
	SeasonWinter	-0.80854	0.19740	**<0.0001**
	Wet.Weight	0.03956	0.01182	0.000832
Paramyxid – Reduced Model	SiteSB	0.59537	0.48503	0.220
	SeasonSpring	-0.73969	0.69938	0.290
	SeasonSummer	0.28834	0.53717	0.591
	SeasonWinter	0.34402	0.53766	0.522
	SexM	-0.36564	0.55897	0.513
	SexU	-0.08357	0.45436	0.854
Microsporidian	SeasonSpring	-18.52201	1604.08	0.9907
Reduced Model				
	SeasonSummer	-2.28730	0.78663	0.003
	SeasonWinter	-18.21641	1621.963	0.991
	SexM	1.13966	0.70929	0.108281
	SexU	0.34739	0.52682	0.509
	Wet.Weight	0.08569	0.2388	**0.000341**

4.4.3 Phylogenetic Analysis

Readable sequencing data was not generated from microsporidians (n=10) and paramyxid (n=25). PCR- derived amplicons, either by forward or reverse reactions. Readable sequencing data was achieved from PCR assay used for haplosporidian (n=217) detection, BLASTn searches and filtering using the NCBI database, no matches to haplosporidians were obtained. Instead, for all readable sequences, hits returned as a high fidelity matches to non-target *C. fornicata* DNA.

4.4.4 Histology

All limpets in which DNA extracts contained 'positive' signals for the PCR based assays above were screened via multi-tissue histology, with an additional selection of limpets to ensure at least 9 individuals per site per month were screened, yielding n = 343. Overall, very few signs of disease or tissue damage were observed in *Crepidula fornicata*.

Gills

Gills are fan-shaped, and their insertion into the mantle is in the left anterior region (Figure 4.6A). *Crepidula fornicata* gills were free from any microbial biofilms or epibionts (Figure 4.7A). The distal region (tip) of each filament is bulbous in nature. The gills have a regionally ciliated epithelium (Figure 4.7B), with an internal haemal space, with muscle supporting the structure of the gills (Figure 4.7C), and a large underlying haemolymph pool is also observable (Figure 4.7D). One individual (MH 44 Oct) was observed to have an unidentified turbellarian free in the gill chamber (Figure 4.8). The presence of this turbellarian did not appear to affect the gill structure of the individual.

Muscle

Muscular tissue in *Crepidula fornicata* is formed by bundles of aligned muscle fibres (Figure 4.9). Overall, very few pathologies were observed in this tissue. One case of an encysted parasite, potentially a digenean trematode, was observed in the muscular tissue of a single limpet (MH 16 May; Figure 4.10A). The only other pathology noticed was a case of damaged muscle that was surrounded by a haemocyte sheath and underlying healthy muscle – no aetiological agent was apparent (SB 36 Dec; Figure 4.10B).

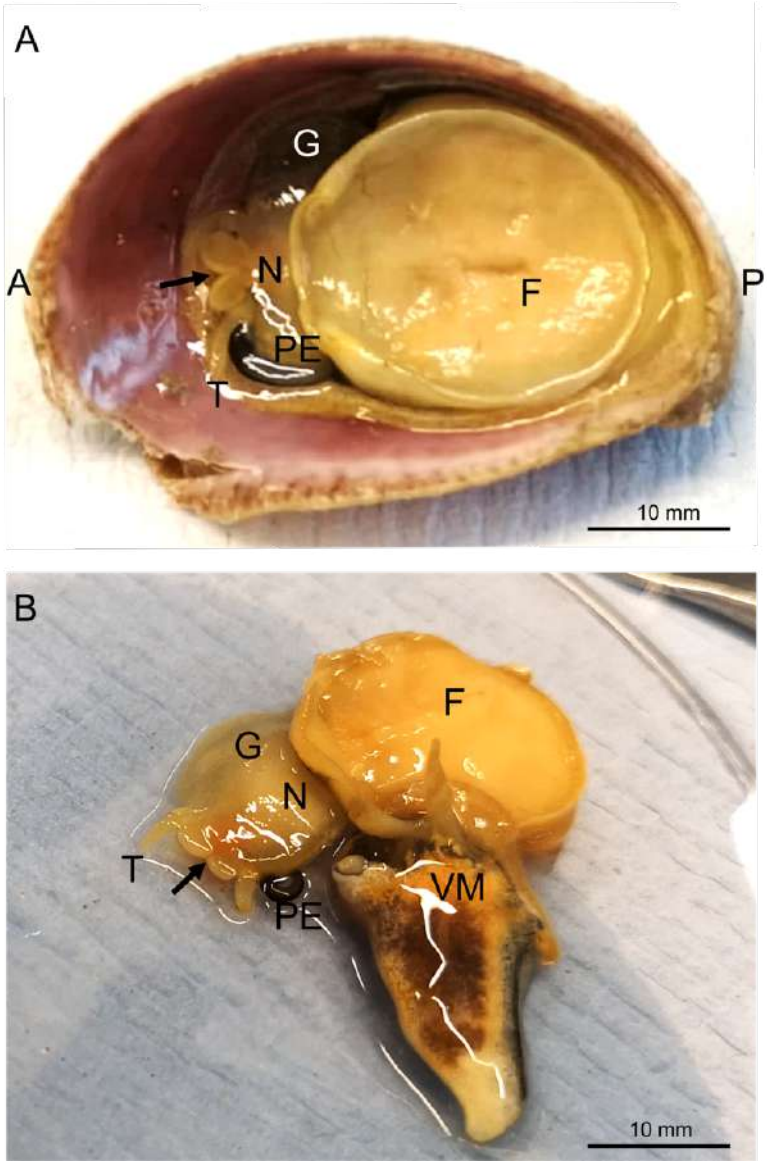

Figure 4.6 (A). Ventral view of *Crepidula fornicata* (A) anterior; (P) posterior; (F) foot; (N) neck; G (Gill), (T) tentacle; (Arrow) mouth; (PE), penis. (B). *Crepidula fornicata* removed from shell. (F) foot; (N) neck; G (gill); (T) tentacle; (Arrow) mouth; (PE), penis; (VM) visceral mass containing the internal organs (mainly digestive tract, digestive gland and gonads).

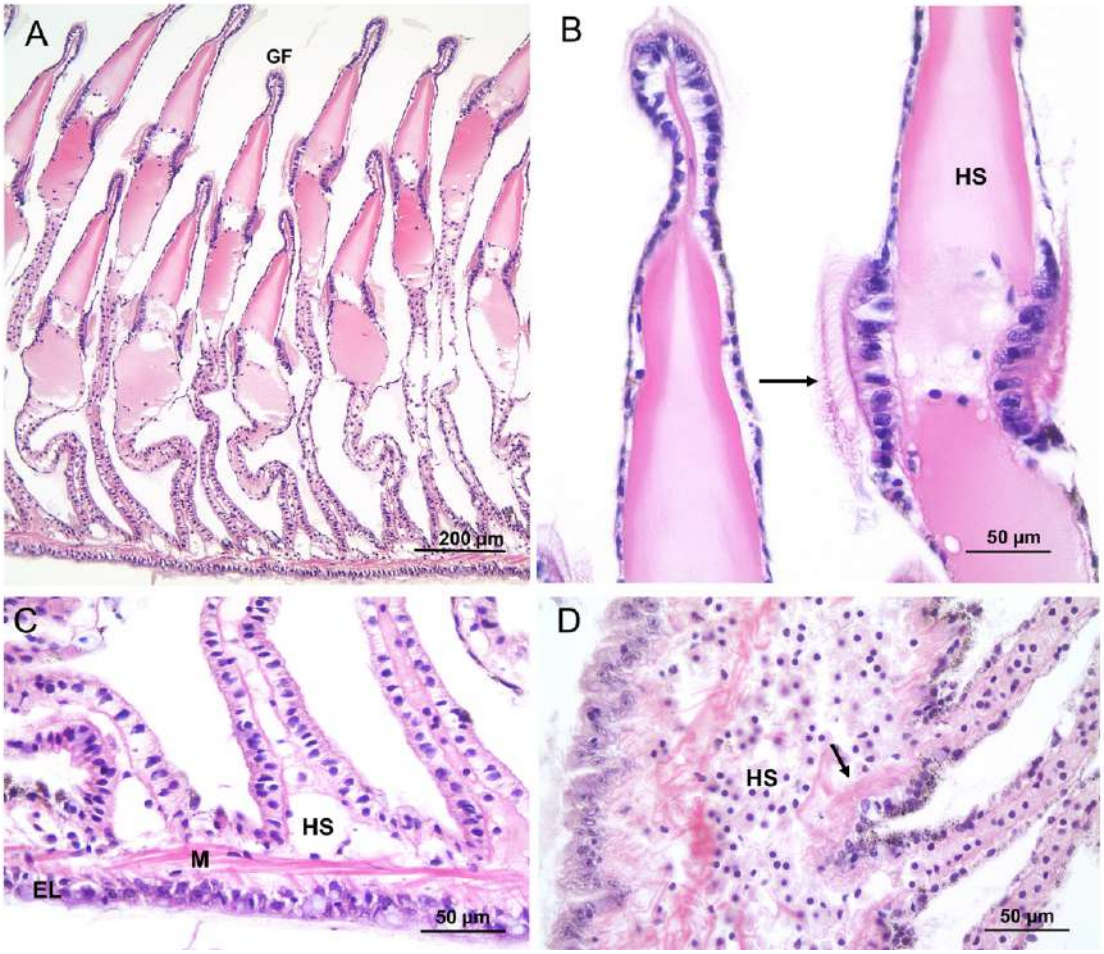

Figure 4.7: (A) Low power micrograph showing the gill structure of *Crepidula fornicata* (GF) Gill filament – Individual MH 6 June. (B) High power micrograph showing the tip of the gill surface (HS) haemolymph sinus, arrow points to cilia – Individual MH 6 June. (C) High power micrograph showing the base structure of the gill in *C. fornicata* (EL) Epithelial layer, (M) Muscle, (HS) Haemolymph sinus – Individual MH 6 June. (D) Large blood (haemolymph) space that runs along the base of the gills, arrow points to muscle supporting the base of the gill – Individual MH 9 June.

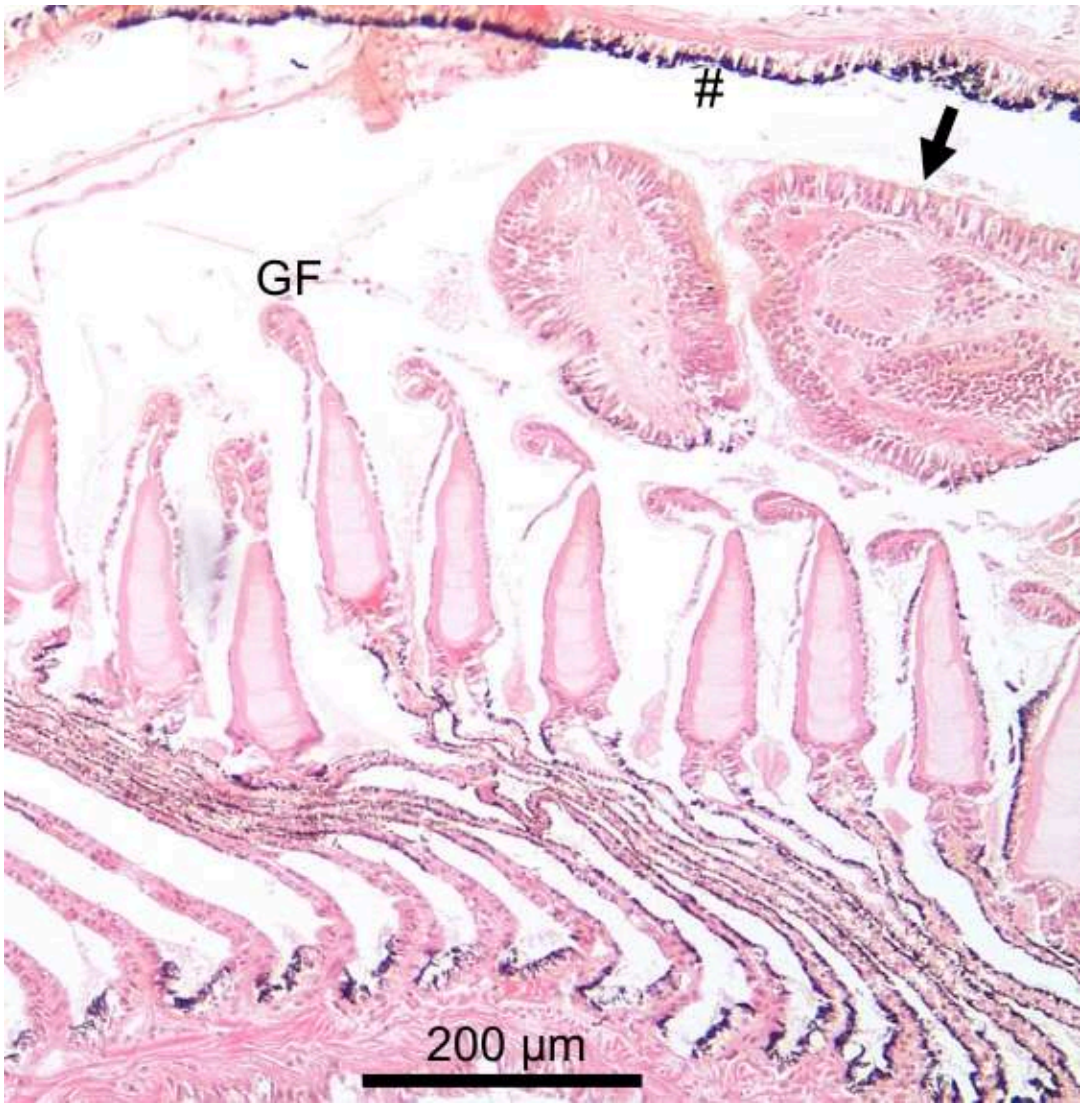

Figure 4.8: Turbellarians present in the gill chamber area (arrow) of *Crepidula fornicata*, (GF) Gill filament, (#) Melanin deposits in the epithelium - Individual MH 44 October.

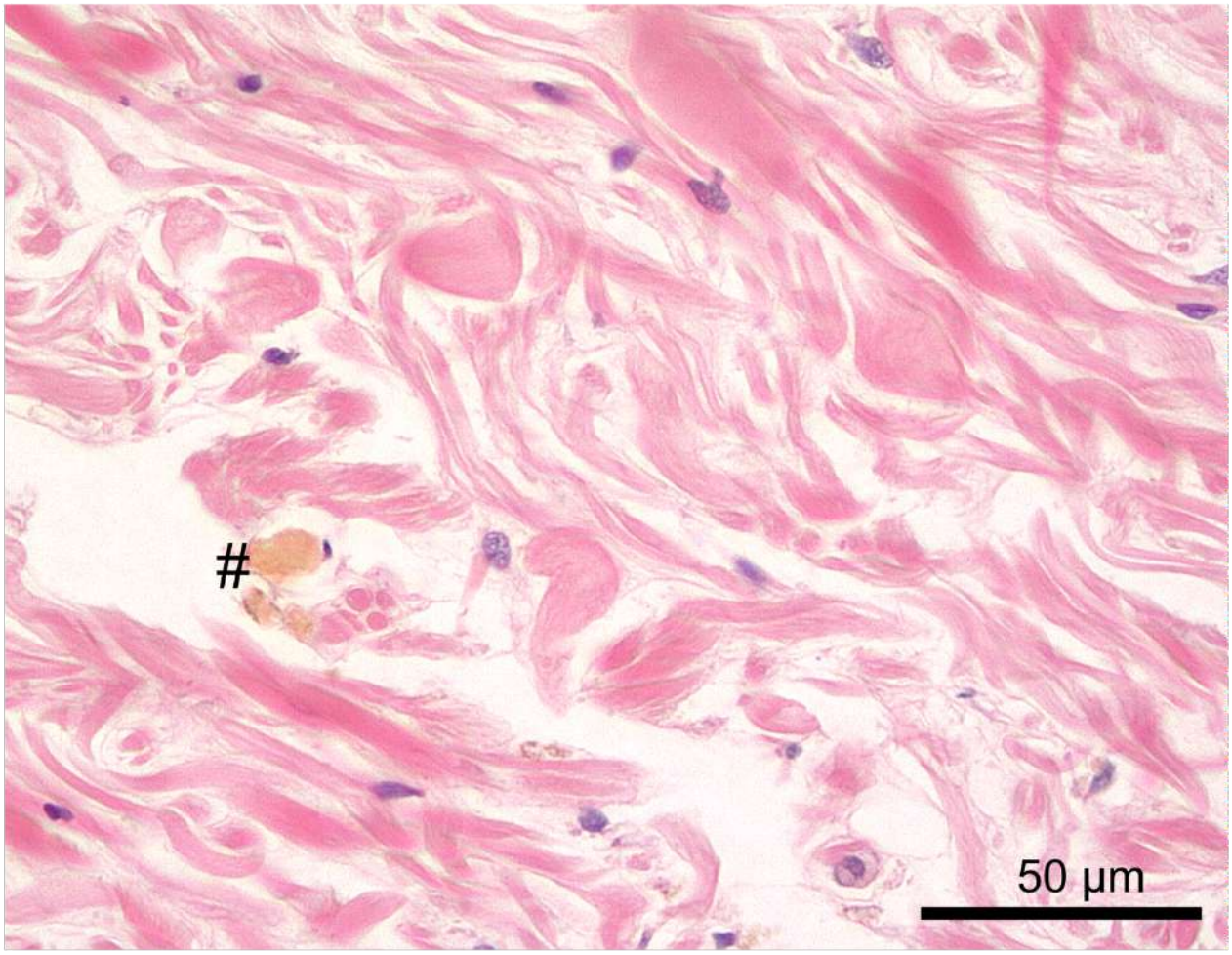

Figure 4.9: Photomicrograph demonstrating the alignment of potentially healthy muscle fibres in *Crepidula fornicata*, (#) Lipofuscin-like material – Individual MH 23 February.

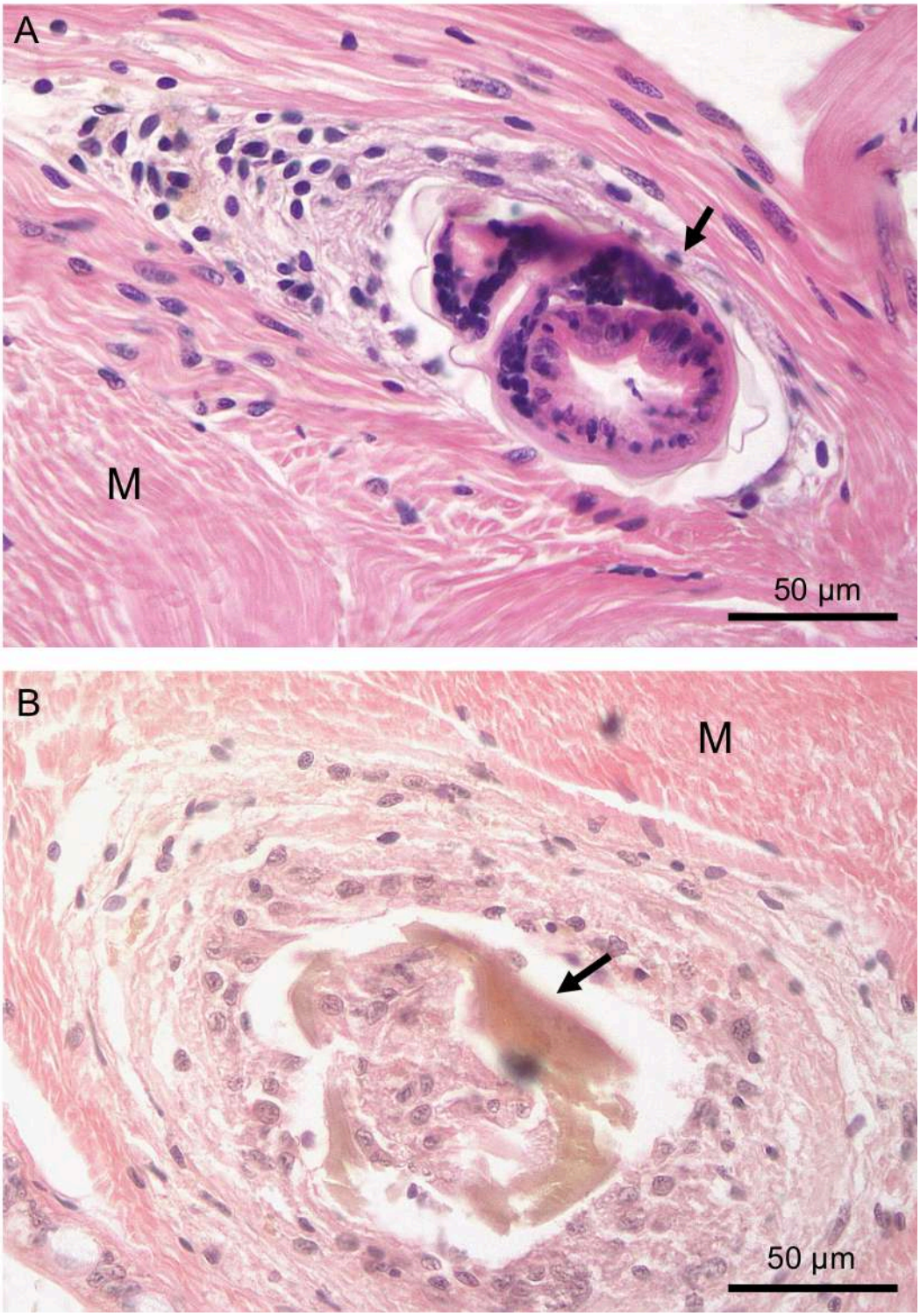

Figure 4.10: (A). Encysted parasite, potentially a digenean trematode (arrow) in the muscle (M) of *Crepidula fornicata* – Individual MH 16 May. Note haemocyte sheath surrounding the parasite. (B). Ensheathed necrotic muscle (arrow) surrounded by haemocyte sheath and adjacent healthy muscle tissue (M) - Individual SB 36 December.

Kidney

The *Crepidula fornicata* kidney is located towards the posterior region of the individual (Figure 4.11). The kidney consists of columnar epithelial cells forming tubular arrangements. No pathologies were observed in this organ.

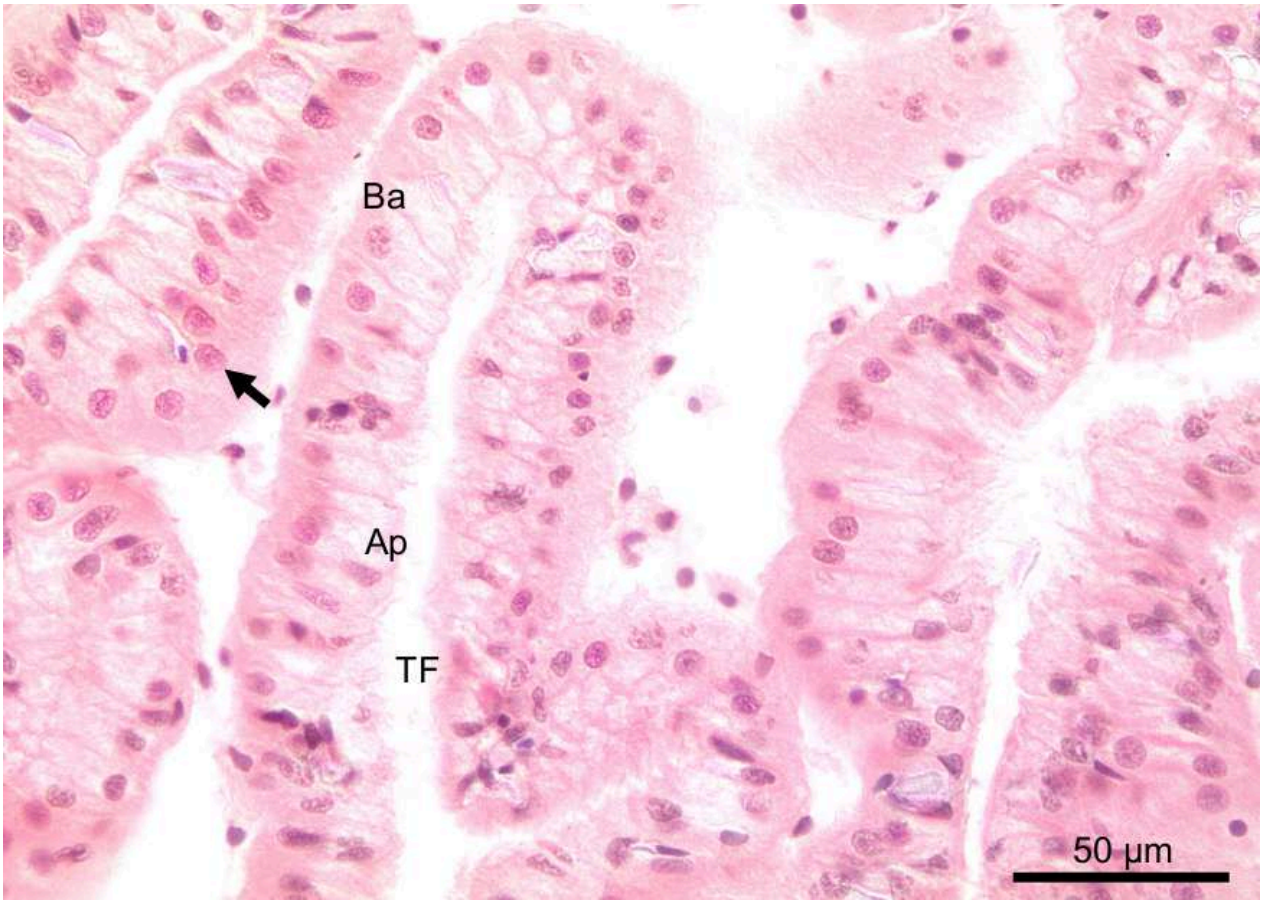

Figure 4.11: Kidney structure of *Crepidula fornicata*, (Ba) Basal surface, (Ap), Apical surface, (TF) Tubular fold, arrow points to tubular cell nuclei – Individual MH 72 December.

Digestive tract

As a general rule for molluscs, at the beginning of the digestive tract (i.e. foregut, closer to the "mouth" of an individual), cells appear cuboidal in shape, and the luminal space tends to be of a smaller diameter (Barnabe, 2018; Harris *et al*., 1998). As it progresses toward the midgut, cells tend to appear columnar in shape, and the luminal space is larger – this is the area where most of the mechanical digestion of food takes place. This variation in structure could be seen in the digestive tract of *Crepidula fornicata* (Figure 4.12A). The anterior digestive tract had a compact luminal space when compared to that

of the mid-digestive tract. Ciliated columnar cells lining the gut epithelium can be seen, along with a layer supporting muscle (Figure 4.12B). Amorphous "balls" of food were present in the digestive tract (Figure 4.12B).

Some pathologies were evident in the digestive tract of *Crepidula fornicata* (Figure 4.13A). Cellular debris (presumably from the epithelium) was seen in the digestive tract of 3/343 (< 1%) of individuals from Swansea Bay (SB Feb 33, SB 3 Aug, SB 66 Sep) but there was no evidence of damage to the tract itself (Figure 4.13A). Turbellarians were observed in the digestive tract lumen of 58 individuals in total (~ 16% of limpets): 45 (13%) individuals from Milford Haven and 12 individuals from Swansea Bay (3%), but there was little evidence of any interaction with the host (Figure 4.13B). These unidentified turbellarians contained a ciliated integument (Figure 4.13B). Some unusual cells were present in the digestive tract epithelium of 11/343 (~ 3%) *Crepidula fornicata* (Figure 4.14). These cells had distinct, condensed chromatin banding in the nuclei and an eosinophilic cytoplasm. The cells were usually surrounded by a vacuolar space, and in some cases, these were seen in continuity with the gut lumen (Figure 4.14A), and free within the lumen (Figure 4.14B). A possibility for the identity of these cells is that they are altered host cells or virus- infected cells, given the appearance of a Cowdry body with emargination.

Digestive gland

The digestive gland of *Crepidula fornicata* is nestled around the digestive tract (Figure 4.12A). The tubules are surrounded by intertubular tissue (connective tissue, haemolymph, cells containing melanin and/or lipofuscin; Figure 4.15A, B). Digestive gland tubules varied in both overall size as well as the luminal space (Figure 4.15A), and contained variable amounts of pigment (entirely likely to be lipofuscin). An example of cellular debris present in the digestive gland lumen was observed in individual 'SB 68 August' but no alteration to the tubule epithelia was noted in this animal (Figure 4.16A). Overall, only 3 limpets sampled showed such damage. Rarely (~ 0.5 % of limpets), tubule damage was encountered (Figure 4.16B), with tubule architecture appearing necrotic and displaced. Occasionally (<1 %) haemocyte infiltration into the intertubular space without any evidence of tubule damage was observed (Figure 4.16D). Figure 4.16E

depicts an encapsulated necrotic tubule with extensive infiltration of haemocytes into the surrounding intertubular space. The potential cause of this is unknown.

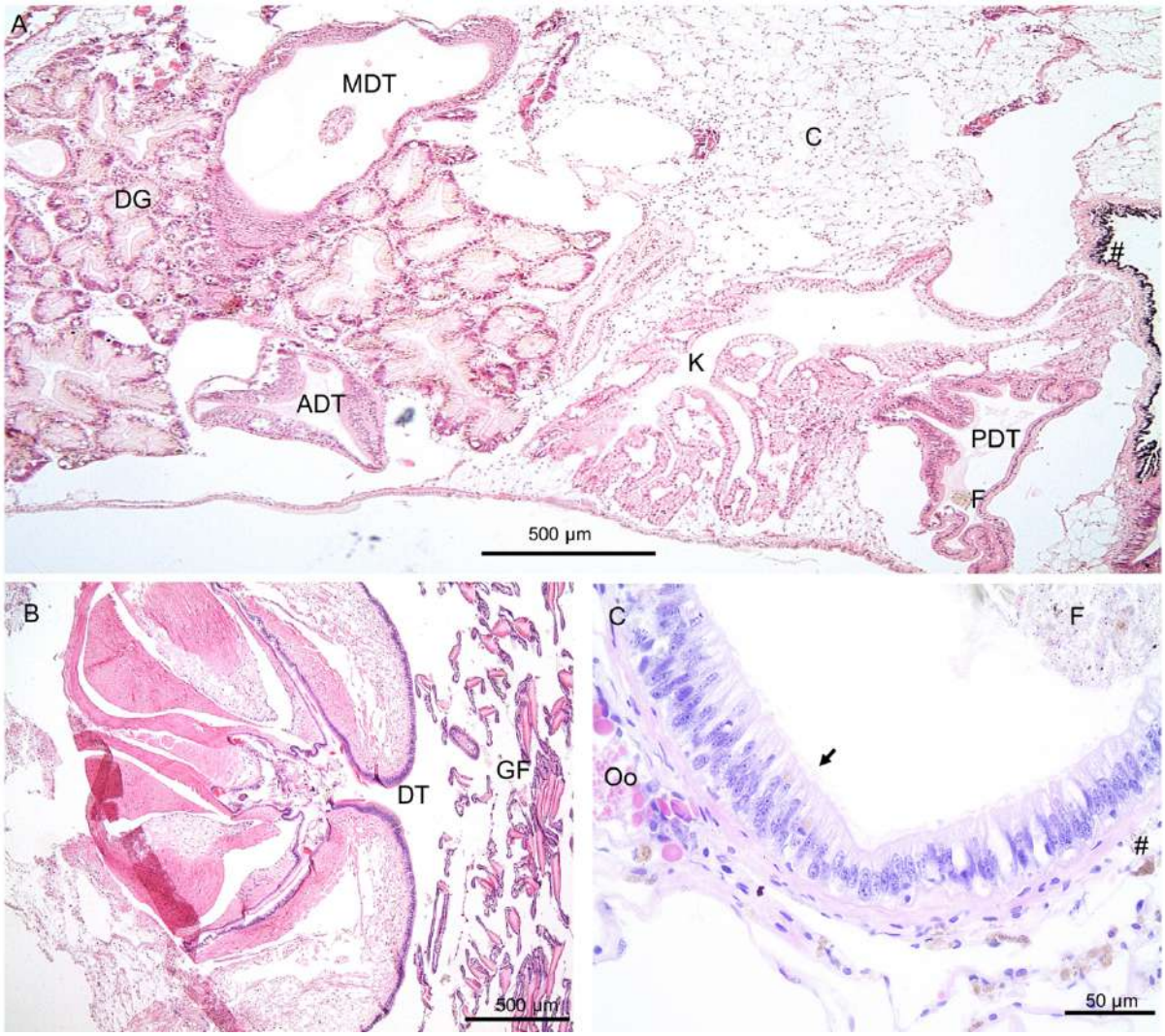

Figure 4.12: (A). Low power micrograph showing the internal anatomy of *Crepidula fornicata* (ADT) anterior digestive tract, (MDT) mid-digestive tract, (PDT) posterior digestive tract, (DG) digestive gland, (K) kidney, (F) food bolus (C) connective tissue, (K) kidney, (#) Melanin deposits – Individual MH 16 May. (B). High power image showing the beginning of the *C. fornicata* digestive tract (DT), (GF) Gill filaments– Individual MH 9 June. (C). High power image showing the lower region of the digestive tract, ciliated (arrow) columnar cells lining the gut epithelium, (Oo) Oocytes, (F) Semi-masticated food bolus – Individual MH 22 January.

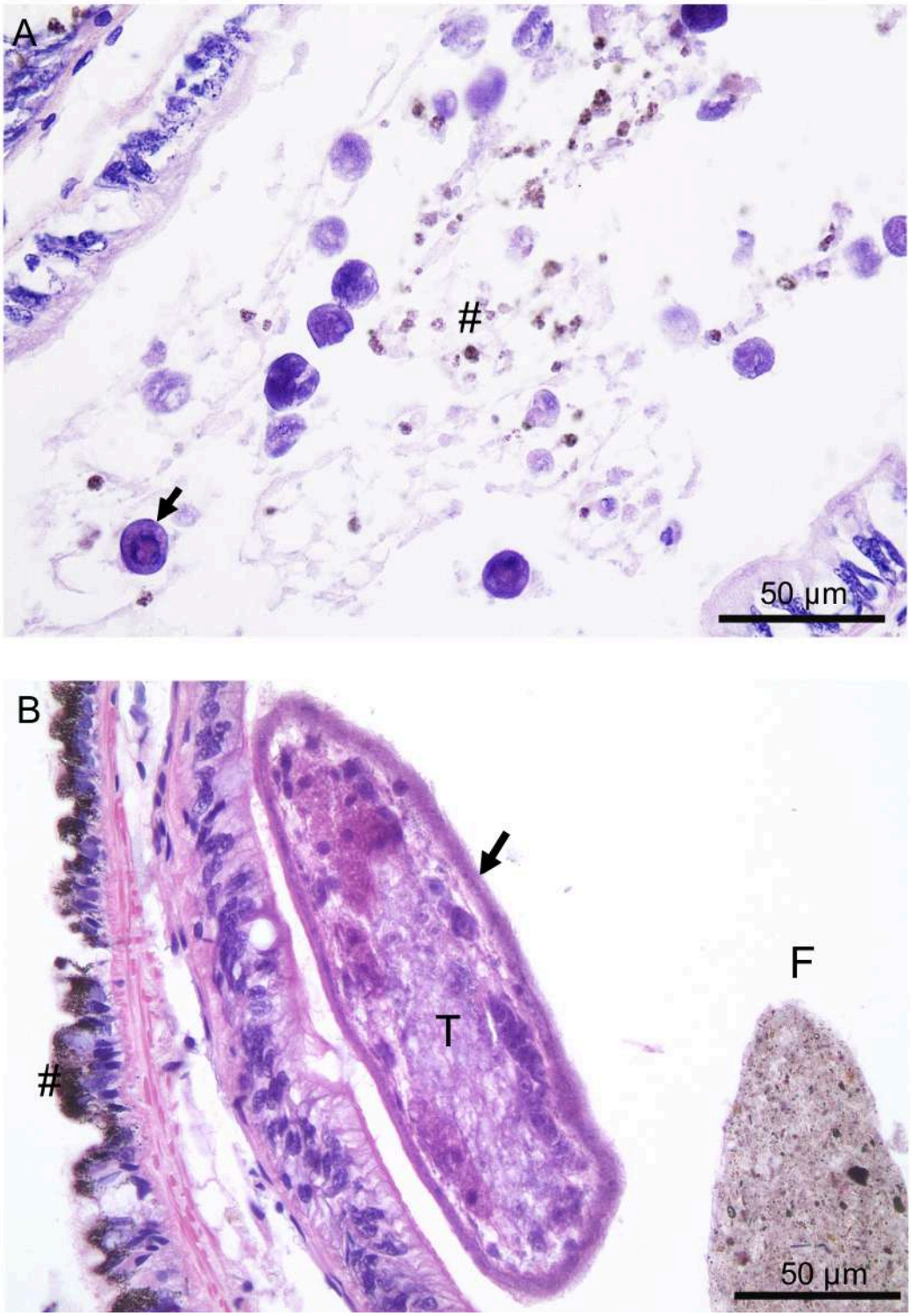

Figure 4.13: (A). Cellular debris present in the GI tract of *Crepidula fornicata,* (#) area of melanin and lipofuscin in the lumen. Arrow points to unusual, larger cells than those present in the epithelium – Individual SB 33. (B). Turbellarian present in the GI tract of *C. fornicata* (T), arrow points to the ciliated integument, (F) Food bolus, (#) Melanin deposits in epithelia – Individual MH 4 June.

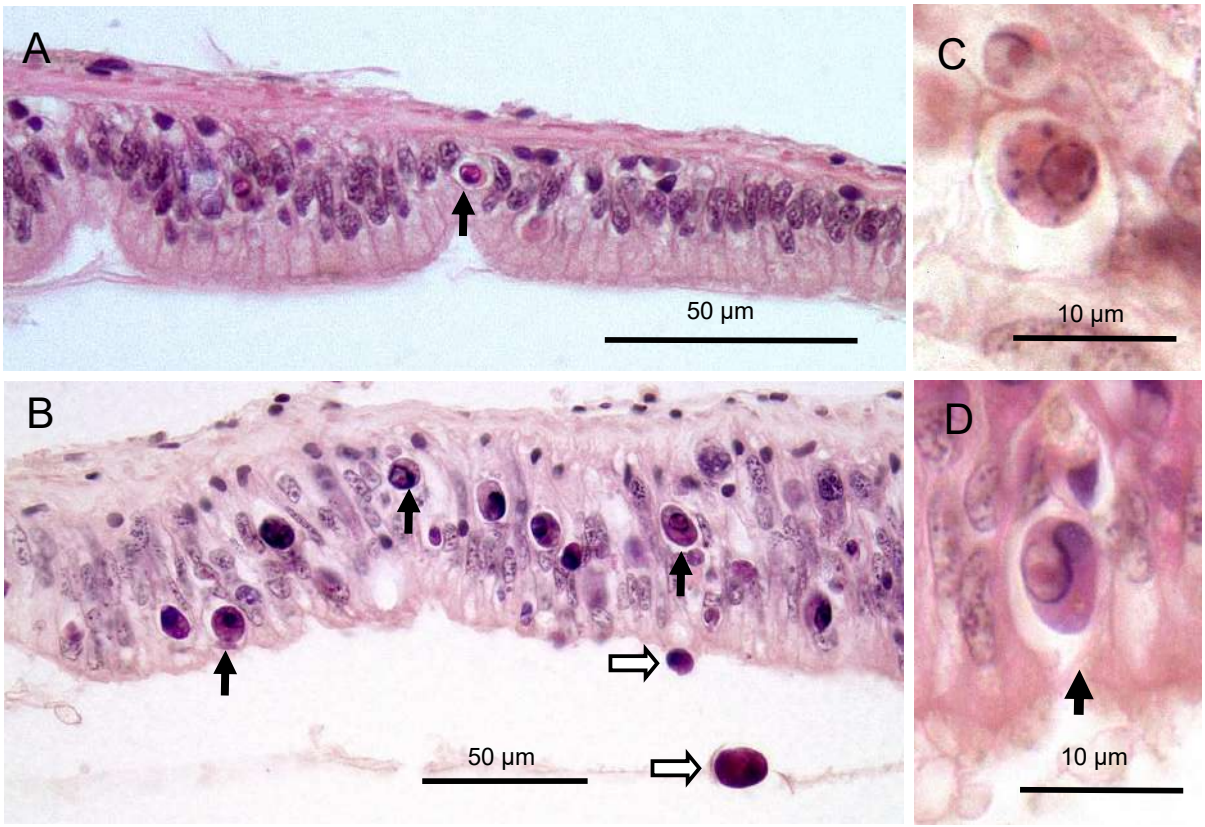

Figure 4.14: (A and B). Photomicrographs showing unusual cells (black arrows) present in the epithelia and lumen of *Crepidula fornicata*. White arrows point to unusual cells in the lumen of the digestive tract – Individual SB 33 March. (C and D). High power photomicrographs of unusual cells found to be present in the epithelial cells of some *C. fornicata* individuals. Note the vacuolar space around these cells and continuity of some of these cells with the gut lumen (black arrow).

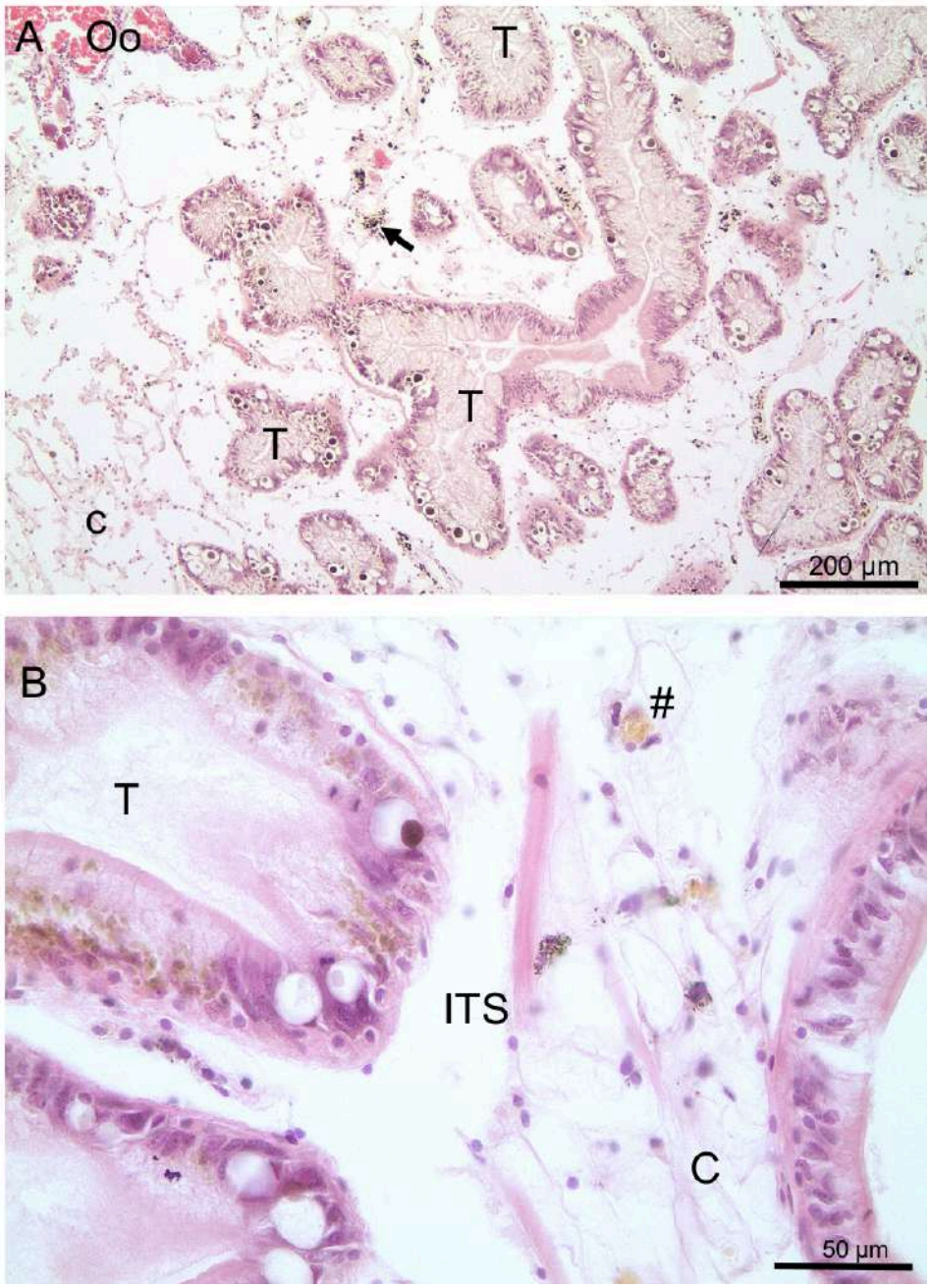

Figure 4.15: Digestive gland of *Crepidula fornicata*. (A). (A). Low power photomicrograph of the digestive gland of *Crepidula fornicata* with varying tubule (T) sizes, (c) intertubular connective tissue, arrow points to areas of melanin, (Oo) oocytes – Individual MH 18 December. (B). High power photomicrograph of tubule (T), (ITS) intertubular space containing (C) connective tissue. (#) Area of lipofuscin-like melanin – Individual MH 41 December.

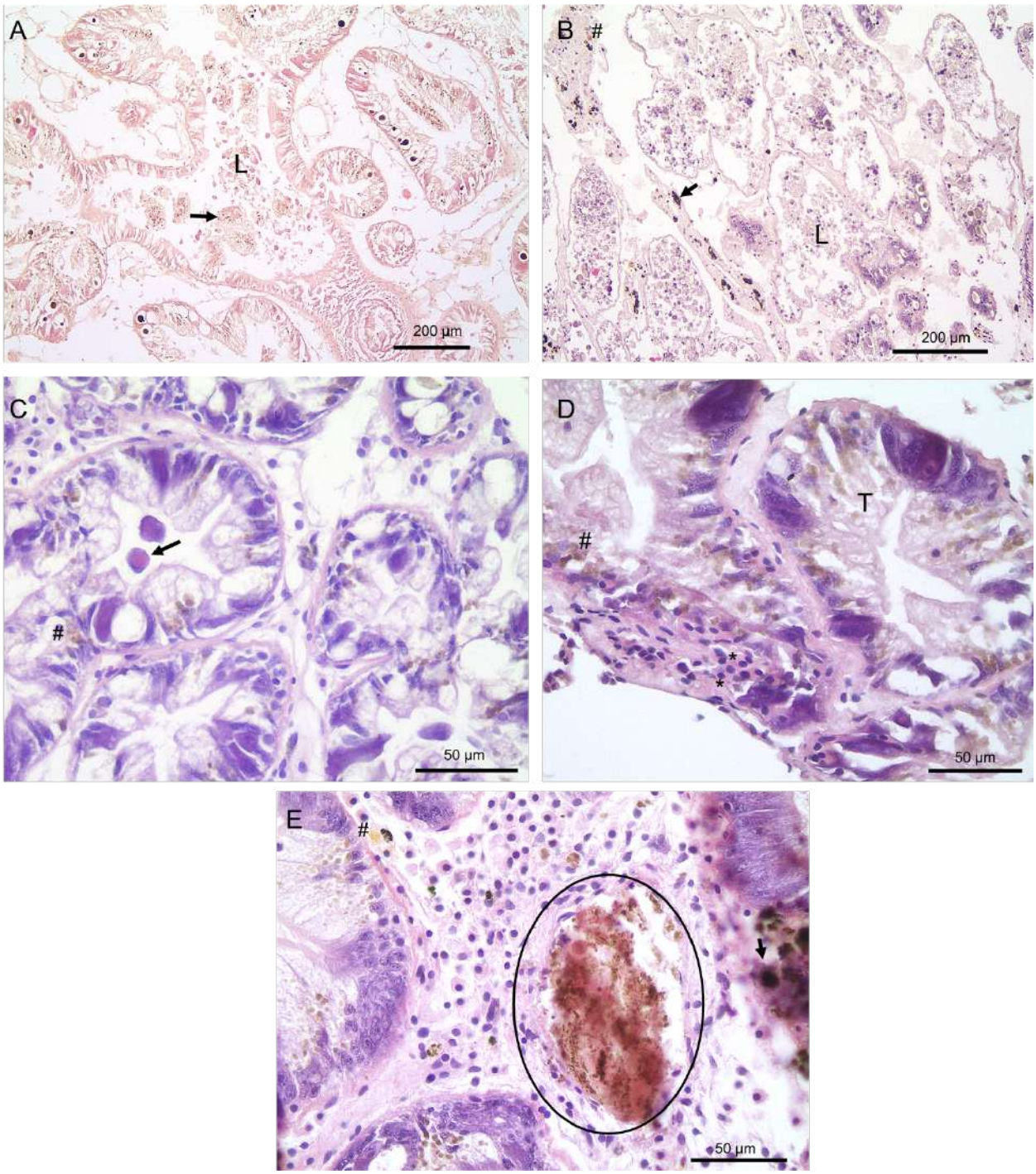

Figure 4.16: Various forms of damage in digestive gland. (A). Low power photomicrograph showing cellular debris (arrow) in the lumen (L) of a digestive gland tubule – Individual SB 68 August. (B). Low power photomicrograph showing tubule (T) damage in the digestive gland of *Crepidula fornicata,* (#) lipofuscin-like melanin deposits – Individual MH 40 May. (C). Unusual cells (arrow) in the lumen of a digestive gland tubule, (#) lipofuscin-like melanin deposits – Individual S.B. 49 February. (D). High power photomicrograph showing haemocyte (asterisk; *) infiltration around a digestive gland tubule (T), (#) lipofuscin-like melanin deposits – Individual MH 43 Jun. (E). Circle area shows an encapsulated digestive gland tubule with a large amount of haemocyte infiltration in the surrounding intertubular space, (#) lipofuscin-like material, black arrow points to melanin deposits – MH 9 June.

Heart

The heart of *Crepidula fornicata* (Figure 4.17A) showed no signs of any pathology. It was observed to contain muscle fibres embedded in haemolymph, with free haemocytes also present (Figure 4.17B).

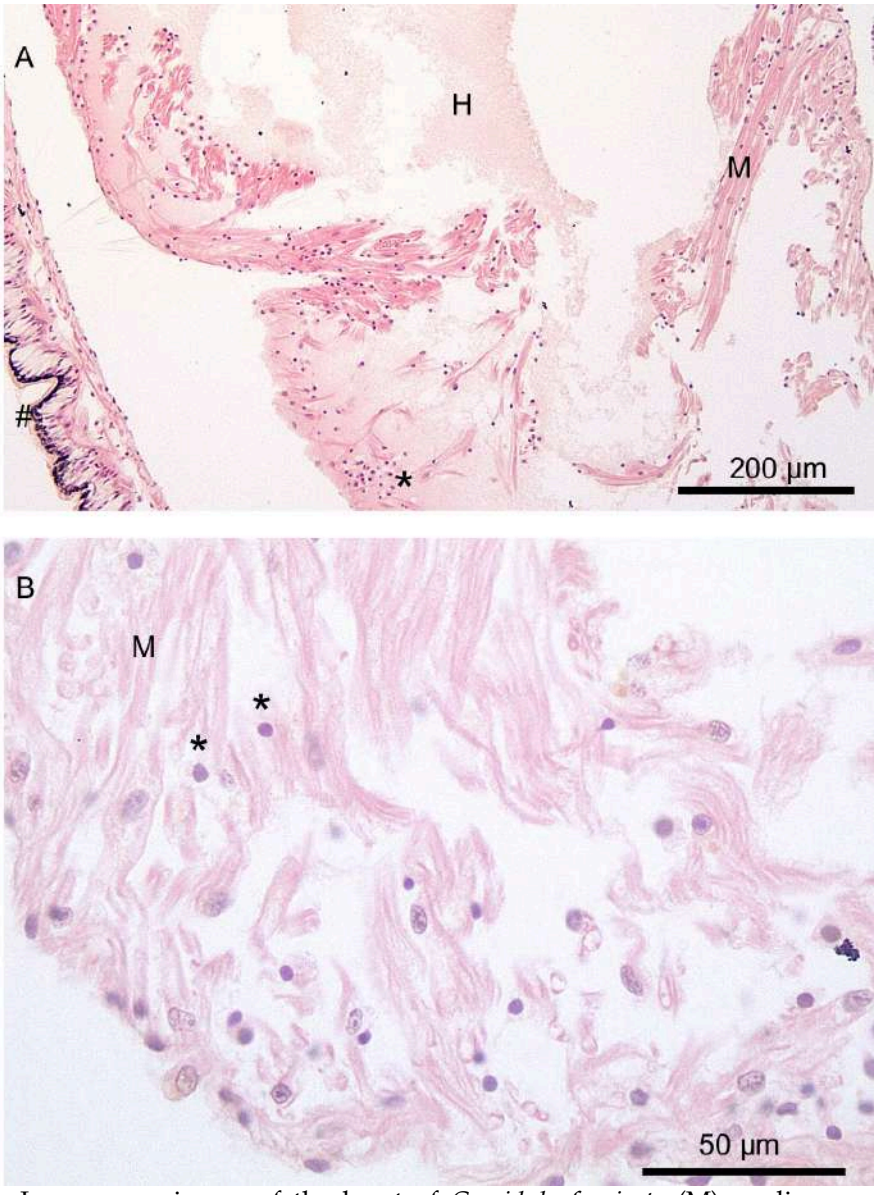

Figure 4.17: (A). Low power image of the heart of *Crepidula fornicata* (M) cardiac muscle, asterisk (*) haemocytes, (H) coagulated haemolymph, (#) melanin deposits in epithelial cells – Individual MH 41 December. (B). High power image of *C. fornicata* heart, (M) Muscle, asterisks (*) indicate individual haemocytes – Individual MH 63 August.

Gonadal tissues

All stages of sperm production were observed in male *Crepidula fornicata.* At the beginning of the cycle, only immature sperm are seen (Figure 4.18A). As sperm production progresses, a mixture of immature and mature sperm could be seen (Figure 4.18B). Eventually, mature sperm form most of the sperm present (Figure 4.18C&D). Sperm storage was also evident away from the site of sperm production (Figure 4.18E).

Various stages of egg development were observed in female *Crepidula fornicata* ranging from primary oocytes that contain a prominent nucleus with a nucleolus, and no cytoplasmic yolk (Figure 4.19C) through to mature, yolky oocytes (Figure 4.19A, B). Empty egg chambers were visualised following their release in late summer and autumn (Figure 4.19C). Such individuals showed reduced numbers of mature eggs and prominent immature eggs. Lipofuscin-like pigment was sometimes found in the gonadal tissue of such animals, perhaps representing a cleansing of the organ (Figure 4.19C). Examples of apparently mature oocytes (as judged by the presence of yolky granules) were found in limpets from all months.

Three limpets (0.17%) collected during the year-long survey were found to have gonadal tissue infected with a cercariae (Figure 4.20). Two individuals were sampled from Milford Haven during August 2019 (MH 4 and MH 29). One infected individual was found from Swansea Bay in October (SB 54). In specimens with a trematode infection, eggs were found to be lost and replaced by the developing parasites (MH 29 Aug; Figure 4.18B). These parasites consisted of sporocysts containing developing cercariae Figure 4.20D). The developing cercariae present in the gonadal tissue could be seen to have tail-like regions (Figure 4.20E). A haemolymph preparation from MH4 August was found to contain free-swimming cercariae in a video taken during routine sampling.

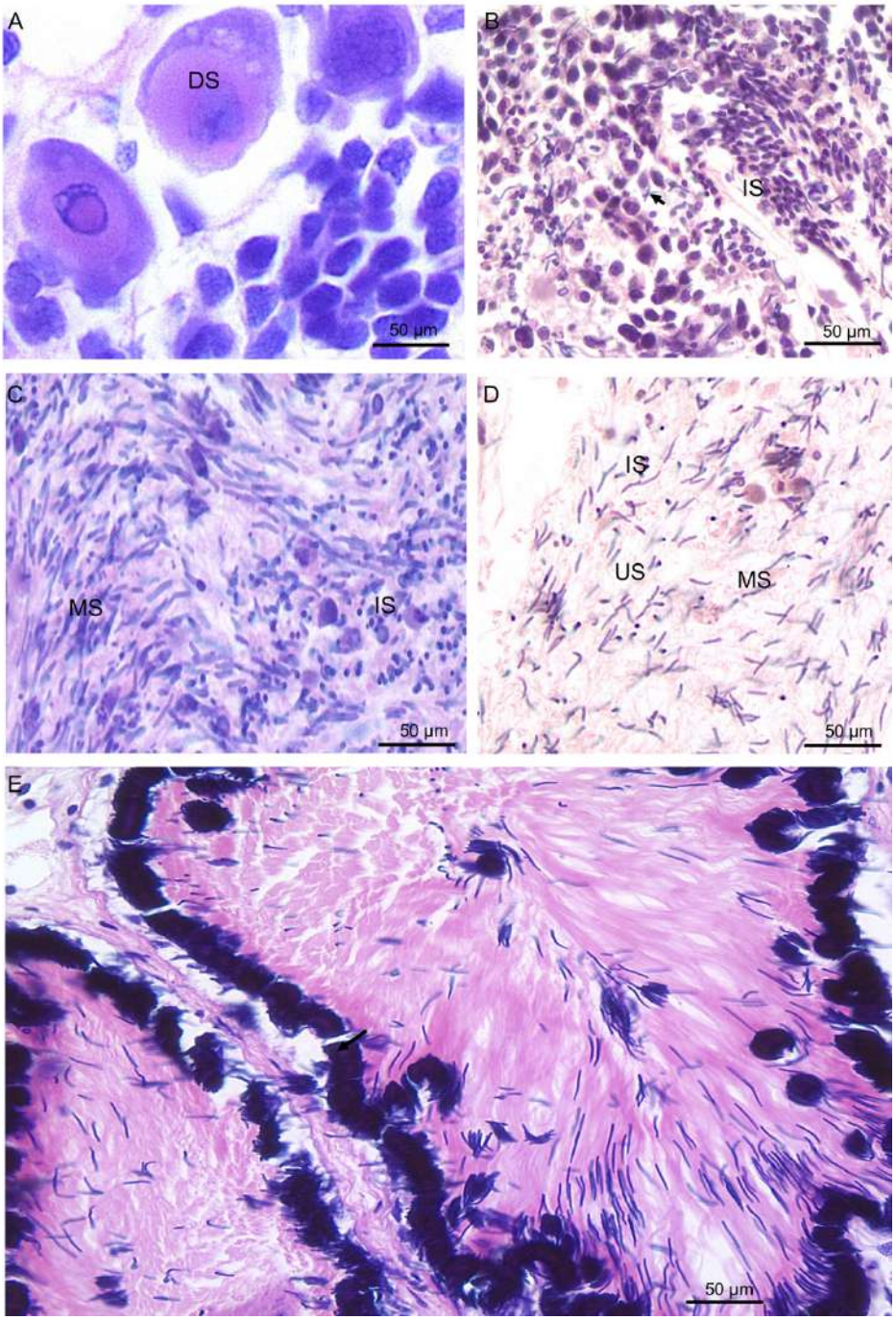

Figure 4.18: (A). Developing sperm in a male *Crepidula fornicata* – Individual MH 1 January. B). Immature male gonadal tissue with the majority of spermatozoa being immature (IS), arrow pointing to some mature sperm – Individual SB 69 June. (C). Male testis with large numbers of mature sperm (MS), and some immature sperm (IS) – Individual SB 16 December. (D). Testis with a mixture of (MS) mature sperm and (IS) immature spermatozoa. (E). Sperm storage in *C. fornicata* – Individual MH 39 April.

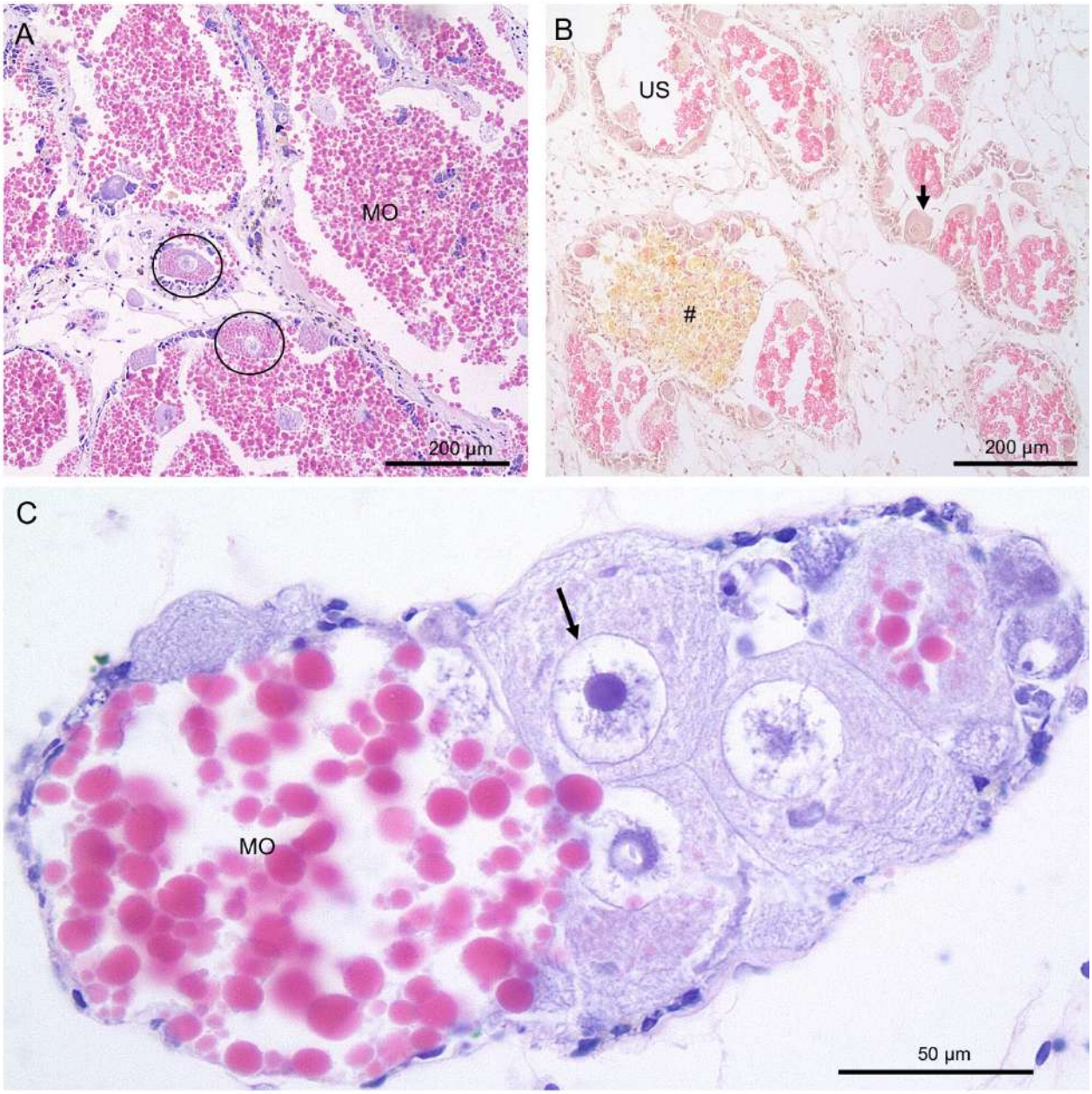

Figure 4.19: (A). Female gonadal tissue of *Crepidula fornicata* with a large number of mature eggs (MO) replete with yolk and some immature eggs also present (circled) – Individual SB 10 January. (B). Large amounts of unoccupied space (US) in the egg chamber probably following the shedding of eggs. Arrow points to developing primary oocyte without yolk, lipofuscin-like material is also present (#) – Individual SB 70 July. (C). Female gonad containing primary oocytes (arrow) with prominent nucleoli, as well as a mature egg (MO) – MH 13 March.

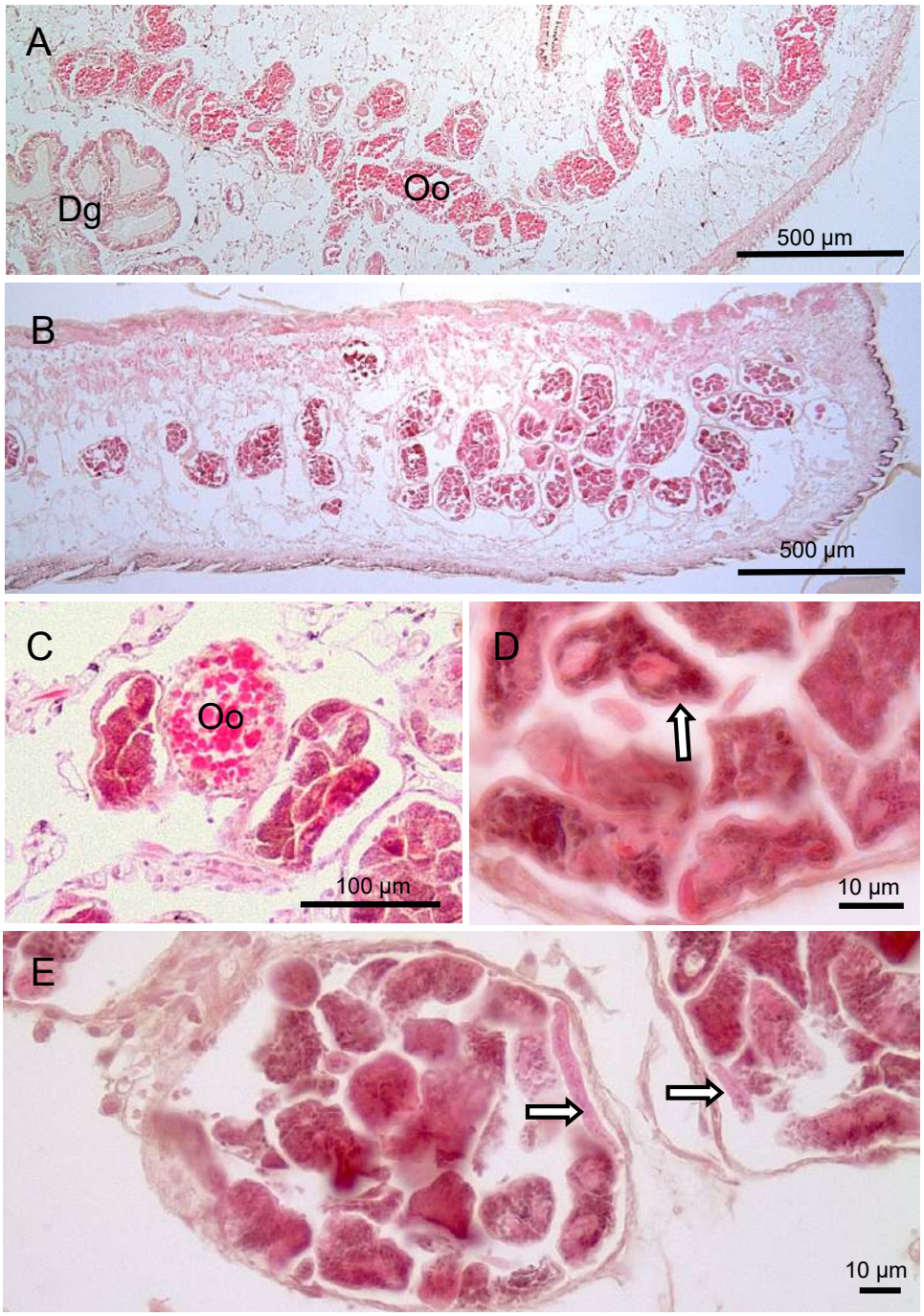

Figure 4.20:(A). Low power micrograph showing normal gonadal tissue in the slipper limpet, *Crepidula fornicata* from Milford Haven in August 2019 showing large numbers of mature oocytes (Oo) containing eosinophilic yolk granules, adjacent digestive gland (Dg) – Individual MH 24 August. (B). Low power micrograph of *C. fornicata* gonadal tissue infected with trematode-like parasites developing within this tissue. Note lack of oocytes. Individual MH 29 August. (C). Region of gonadal tissue in an infected slipper limpet (MH 4) showing a single oocyte (Oo) remaining surrounded by developing parasites. (D). High power micrograph showing the appearance of developing parasites. Note amorphous thin cuticle (arrow) surrounding these. (E). Micrograph of developing parasites in gonadal tissue showing tail-like region (arrows).

4.4.5 Factors associated with parasites observed via histology

Out of the 343 individuals screened via whole tissue histology, a total of 94 samples were seen to contain parasites/symbionts. These parasites fall broadly into two categories, the first being a potential apicomplexan-like parasite seen embedded in the epithelial wall of the GI tract and the other being a turbellarian. The presence of a gonadal trematode infection was observed in a small percentage of individuals (3/94; < 1%). However, due to the small sample size, it was not possible to statistically determine any potential factors associated with its presence. It should be noted that all three individuals with gonadal trematode infections were female. To determine if any factors may be contributing significantly to the presence of either the apicomplexan-like parasite or the turbellarian, a binomial logistic regression model was utilised Figure 4.3). The reduced model for the apicomplexan parasite showed that season was a significant contributing factor, with Spring (P = 0.0000876) and Winter (P = 0.00012) having the highest prevalence with 18/65 and 27/81 positive samples, respectively. Within stack position was also found to be a contributing factor to the presence of apicomplexans within *Crepidula fornicata*. With regards to the contributing factors to the presence of the turbellarians, season and site appeared to be the most important (Table 4.3). *Crepidula fornicata* sampled during the Summer had the highest prevalence of turbellarians (*P* = 0.02179), with them being observed in 25/86 individuals. The Swansea Bay population of *Crepidula fornicata* had a significantly lower likelihood of carrying a turbellarian (*P* = 0.00109). Individuals with a lower TCBS CFU counts appeared to be less likely to be carrying a turbellarian parasite (*P* = 0.0046*)*. Sex or size within the stack did not have a statistically significant influence on the presence of either the apicomplexan-like parasite or the turbellarian.

Table 4.3: Binomial logistical regression model testing the influence of biometric and environmental predictor variables on the presence of a potential apicomplexan parasite and a turbellarian in a subset of *Crepidula fornicata* sampled from Swansea Bay and Milford Haven

Model	**Predictor Variable**	**Estimate Slope**	**S.E.**	***P*-value**
Apicomplexan Reduced model	Position.In.Stack	-2.993	0.464	**0.04171**
	SeasonSpring	2.115	0.532	**0.0000876**
	SeasonSummer	0.1179	0.6298	0.06216
	SeasonWinter	1.968	0.5056	**0.00012**
	SiteSB	-0.3106	0.3402	0.36201
	TCBS	- 1.426e-06	7.635e-07	0.06270
Turbellaria Reduced model	SeasonSpring	0.2928	0.4462	0.512073
	SeasonSummer	1.850	0.4777	**0.000130**
	SeasonWinter	-2.874	1.030	**0.00558**
	SiteSB	-1.287	0.3177	**0.000721**
	TSA	-4.544e-07	3.177e-07	0.153496
	TCBS	-1.971e-06	6.907e-07	**0.004594**

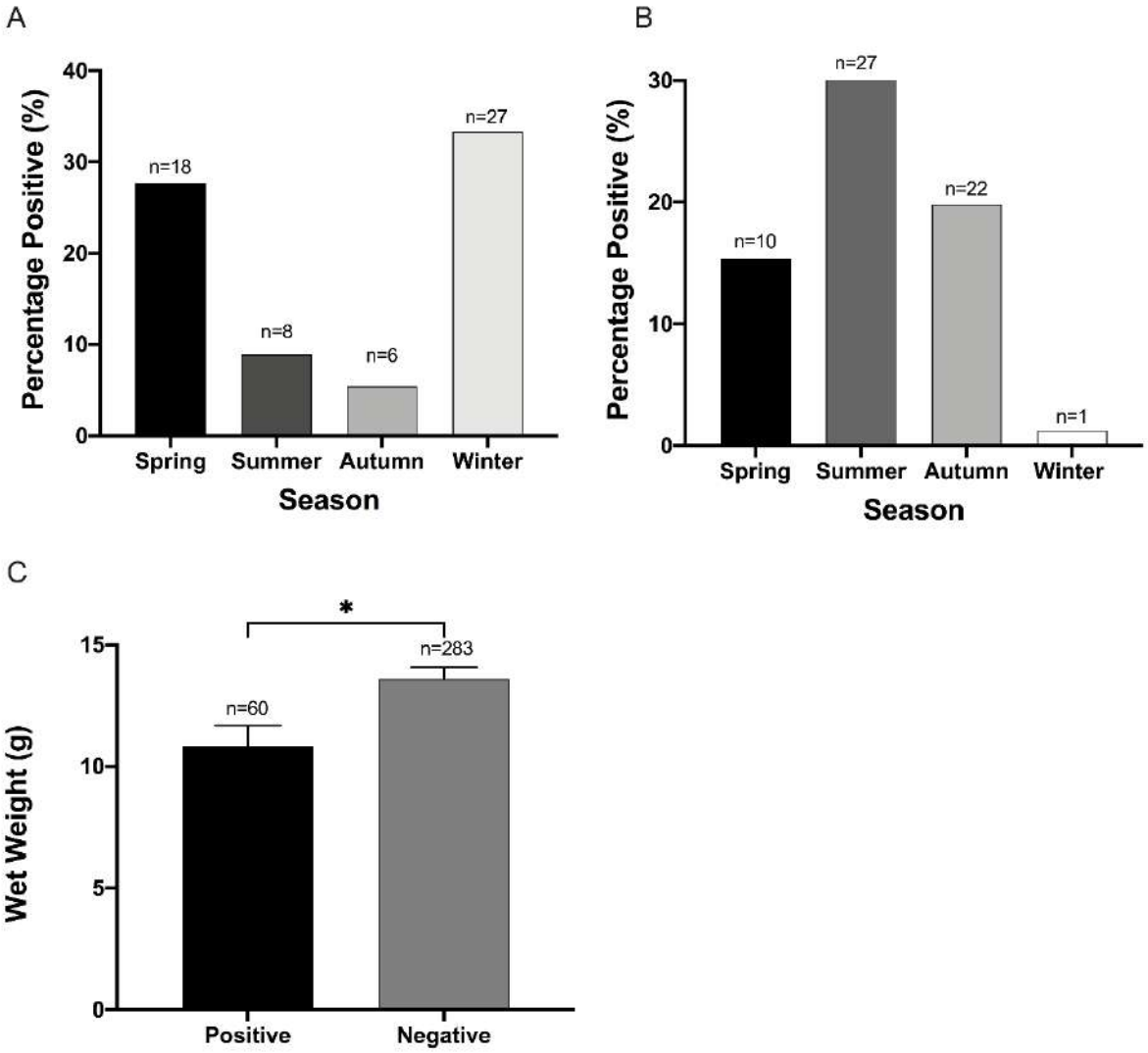

Figure 4.21: Significant variables associated with the presence of potential apicomplexan and turbellarian parasites in *Crepidula fornicata* individuals, determined through binomial logistical regression modelling. (A). Effect of season on percentage (%) of *C. fornicata* individuals that were found to contain what is thought to be apicomplexan parasites sampled during Spring (n=65), Summer (n=86), Autumn (n=111), and Winter (n=81) 2019 across Milford Haven and Swansea Bay. (B). Seasonal variation in the detection of Turbellaria in *C. fornicata* sampled across Spring (n=65), Summer (n=86), Autumn (n=111), and Winter (n=81) 2019 across Milford Haven and Swansea Bay. (C). Effect of site on the number of individuals with turbellarians detected across Milford Haven and Swansea Bay (n=57).

4.5 Discussion

To date, few reports of diseases or pathogens in *Crepidula fornicata* have been described. This chapter aimed to determine the presence or absence of several key disease-causing pathogens in shellfish, notably haplosporidians, microsporidians, and paramyxids. Using PCR-based methods and Sanger sequencing revealed the Milford Haven and Swansea Bay populations of *Crepidula fornicata* to be free of these target pathogens (i.e. microsporidians, haplosporidians, and paramyxids) that infect both bivalves and gastropods. Subsequent histological assessment concluded *Crepidula fornicata* collected from Milford Haven and Swansea Bay were free from the target pathogens, despite the initial PCR results being positive (amplicons of expected size). Unfortunately, there was a high rate of false positives among the PCR products i.e. individuals being PCR positive, but Sanger sequencing revealing no matches to the target diseases. Sequencing results were also supported by histology as no signs of disease or typical immune responses were visualised.

Interestingly, a study carried out by Davies *et al*. (2021) in Swansea Bay found populations of shore crabs *Carcinus maenas,* as well as seawater eDNA samples, to be positive for the same parasite taxa screened for in this chapter. This indicates that although these pathogens are present in the environment and other invertebrates, *Crepidula fornicata* does not appear to be susceptible to them. Though much of the success of *C. fornicata* as an invader is attributed to factors such as its fecundity and dispersal ability (Viard *et al*., 2006; Pechenik *et al*., 2017), this apparent lack of susceptibility to 'local' shellfish diseases could be an important contributing factor to the establishment of slipper limpets outside of their native range. It would seem that once *Crepidula fornicata* is established in an area, there is little in the way of natural controls, i.e., pathogens or parasites, to halt its invasion. Data presented in this chapter provides evidence of *C. fornicata* "parasite escape" in the waters surround south west Wales – however, there is so little literature on *C. fornicata* pathobiology or epizootiology within its native range, it is hard to determine from what the limpet has escaped. Some work is still needed to emphatically identify the parasites present in its native range

The use of PCR is frequently deployed to determine the presence or absence of a specific disease-causing agent in shellfish and other animals (Chen *et al*., 2012; Lynch *et al*., 2013b; Aranguren and Figueras, 2016; Ward *et al*., 2016). In this chapter, based only on PCR results, many samples would have been considered positive for haplosporidians, microsporidians, and paramyxids. However, Sanger sequencing revealed the results from the PCR assays to be false positives. Claydon *et al*. (2004) described the issue of false positives with regards to the PCR protocol for white spot syndrome virus (WSSV), which is a notifiable crustacean pathogen. They found that the amplicon obtained had a non-phylogenetic relationship with WSSV. A study carried out by Jones (2003), re-tested crab samples found to be positive *via* qPCR and concluded that the initial results were false positives. Bryson (2014) conducted an extensive screen for the presence of bacterial endosymbionts in scorpions and found a very high rate of false-positive samples – and again, requested caution in the interpretation of such molecular based assays. These studies highlight the importance of not solely relying on the outcome of PCR assays and demonstrates the importance of using other available methods such as sequencing, haemolymph screens (e.g., Chapter 3), and histology to confirm results (Howard *et al*., 1994; Crossley *et al*., 2020). The misdiagnosis of a pathogen can have harsh consequences, especially when that pathogen is an OIE notifiable disease, e.g. *Bonamia ostrea*, *Marteilia refringens*, and *Perkinsus marinus* (see OIE 2019), as it can result in the incorrect quarantine and needless destruction of animals (Claydon *et al*., 2004), as well as aquaculture or capture fisheries site closures.

Turbellarians were found in some slipper limpets, with no signs of disease and/or tissue damage. Turbellarians can act in both an endocommensal and parasitic nature in molluscs and can freely move between the mantle cavity and alimentary canal of the host (Longshaw and Malham, 2013). They are commonly found in low prevalence and intensities resulting in little harm to the host (Villalba *et al*., 1997; Thieltges *et al*., 2006a). *Urastoma cyprinae* is a commonly found turbellarian in the gills of several mollusc species or found free-living in muddy sediments (Goggin and Cannon, 1989; Rayyan *et al*., 2004; Aguirre-Macedo *et al*., 2007). *Crepidula fornicata* had the highest prevalence of

turbellarians during the summer months, followed by autumn. Several studies have found the highest prevalence of turbellarian infections to occur during the summer and autumn months in species such as the Mediterranean mussel *Mytilus galloprovincialis* and Red sea snails *Nerita* spp (Crespo-González *et al*., 2010; Hassan *et al*., 2017; Bhaby, 2018). Some suggest that turbellarians leave their hosts when the environmental temperature drops, causing lower intensities during colder mouths (Fleming, 1986; Crespo-González *et al*., 2010). A site difference was found with regards to the presence of turbellarians in *C. fornicata,* with those at Swansea Bay having a lower prevalence. A review of the importance of turbellarians in the marine meiobenthos carried out by Martens and Schockaert (1986) found that in subtidal areas such as Swansea Bay, turbellarians were not abundant due to subtidal areas being a less dynamic environment. Grain size and the packing of these grains determine the available space for "interstitial meiofauna", e.g. turbellarians. In muddy/wave protected areas, less space is available for these interstitial meiofauna to exist. Besides the presence of turbellarians in the digestive tract, the only other noted pathology was the presence of some cellular debris, which was concluded to be simply due to the mechanical digestion of food taking place (Lobo-da-Cunha, 2019).

Three females (0.17%) *Crepidula fornicata* from both Swansea Bay and Milford Haven were found to contain gonadal trematodes that caused parasitic castration – an event widely reported in other molluscs (Cheng *et al*., 1973; Rice *et al*., 2006; Brian and Aldridge, 2021). Interestingly, a survey of Rhode Island and Massachusetts populations of *C. fornicata* carried out by Pechenik *et al*. (2001) found that it did not act as a first host for trematodes and the authors suggested that slipper limpets were not parasitised by digeneans. However, their observations were only based on a small sample size (n=136) over a limited temporal range (sampling took place once in June, July, and September 1999) and hence should be treated with caution. Trematodes have often been known to castrate their gastropod hosts (Cheng *et al*., 1973; Mouritsen and Poulin, 2002; Serbina, 2015). Parasitic castration can result in the incomplete or complete disruption of host gamete production and likely allows for the redistribution of metabolites to the trematode (Valderrama *et al*., 2004). In *Crepidula fornicata* infected

with this trematode (Family Microphallidae), eggs were found to be lost and replaced by the developing parasite. Williams and Brailsford (1998) recorded a higher prevalence of parasitic infection by larval digenean trematodes in female *Littorina obtusata,* but found a higher prevalence in male *L. fabalis*. Lambert *et al*. (2016) also recorded a higher prevalence of trematode infection in female *L. littorea*. There are several possibilities for why trematodes may favour infecting some females more so than males. In species such as *Crepidula fornicata,* females tend to be the oldest individual and are found at the bottom of a stack. It has been suggested that generally larger (often older) individuals are more likely to be infected with parasites than smaller individuals due to an increased exposure over time (Matthews *et al*., 1985; Kube *et al*., 2002). Studies have also suggested that female gastropods may have a higher prevalence of trematode infection due to different feeding activity patterns, and/or a decreased resistance to infection (Tétreault *et al*., 2000). The identity of these trematodes was not established in the current study, but subsequent experiments conducted by others using universal PCR primers for trematodes with the haemolymph samples from trematode-infected animals, concluded that these belong to the Family Microphallidae (see Bevan, Coates, Rowley, personal communication). *Microphallus* spp. are parasites of birds and fish which use a variety of gastropods, including littorinids, as intermediate hosts. Miracidia invade these molluscs resulting in the generation of large numbers of cercariae that are shed and infect decapod crustaceans. Within these crabs, they become encysted in the tissues including the hepatopancreas, a develop into metacercariae. The life cycle is completed when infected crabs are eaten by the definitive host. It is possible that *Crepidula fornicata* has become an accidental host for a microphallid parasite in these two locations.

The identity of the unusual 'apicoplexan-like' cells found in the digestive tract epithelia of *Crepidula fornicata* is unknown. Several explanations exist including (1). Altered host (self) cells undergoing cell death, (2). viral infection resulting in abnormal morphology or (3). parasitisation by apicomplexans. Apicomplexans are a group of unicellular, regularly highly pathogenic, obligate parasites that use one or two hosts to complete a reproductive cycle (Kristmundsson and Freeman, 2018). This group of parasites was shown to be mainly responsible for the total collapse of an Icelandic population of

Iceland scallops *C. islandica* (see Kristmundsson *et al*. 2015), and negatively impacted New Zealand oyster populations (Hine, 2002). The prevalence of these potential apicomplexan-like cells in *C. fornicata* was highest during the cooler months, i.e. winter and autumn. Kirk *et al*. (2013) studied the prevalence of apicomplexans in reef corals in Florida and the Bahamas, also finding that prevalence was highest in the cooler months. Kristmundsson *et al*. (2015) noted that a seasonal difference in the prevalence of the apicomplexan infection in the adductor muscle of the Icelandic scallop *Chlamys islandica,* with prevalence higher in spring. Another possibility for the identity of these cells is that they are altered host cells or virus- infected cells, given the appearance of a Cowdry body with emargination. A wide range of viral families have been reported in marine molluscs including Herpesviridae, Papovaviridae, Togavridiae, Retroviridae, Reoviridae, Barnaviridae, and Picornaviridae (Renault and Novoa, 2004; Meyers *et al*., 2010), however, without ultrastructural examination of such cells their identity remains elusive. There are some limitations in diagnostic tools with regards to the identifcation of viruses, traditionally, the diagnosis of viral agents has been reliant on cell culture in which viruses show cytopathic effects and immunoassays based on antibodies having the binding specificity for the target virus (Leland and Ginocchio, 2007), alongside the amplification of specific gene segments of the virus by PCR (Munang'andu *et al*., 2017). A large proportion of viruses in aquatic environments are unculturable (Wang *et al*., 2002), and no available antibodies available for their identifcation, or specific primers for PCR detection, which precluded the study (Munang'andu *et al*., 2017). However the emergence and widespread use of high-throughput sequencing has promoted the field of viral metagenomics, in order to annotate viral taxonomies, identify functional genes, and discover new viruses (Ge *et al*., 2012; Wei *et al*., 2018; Richard *et al*., 2020).

4.6 Conclusion

Results from this chapter have addressed the current lack of knowledge regarding the disease status of the invasive slipper limpet *C. fornicata.* Overall, populations were healthy, with no individuals being deemed positive for any of the target pathogens such as haplosporidians and paramyxids that infect other molluscs of commercial and

economic significance. Whole tissue histology revealed the presence of trematodes, turbellarians, and some distinct cells, which are considered to be apicomplexan-like parasites. It appears that *C. fornicata* may not be susceptible to many of the disease-causing agents known to be present in their vicinity, and this could be an important factor when considering its success as an invasive species in the region. *Crepidula fornicata* in Milford Haven and Swansea Bay do not appear to be acting as a reservoir for any notifiable pathogens of either shellfish and/or humans, which complements the findings of Chapter 3. This chapter further highlights the importance of using a multi-resource approach (e.g., sequencing and histopathology) in determining the disease status of an animal, due to the occurrence of false-positives via PCR analysis alone.

Chapter 5 Modelling the potential spatial distribution of *Crepidula fornicata* under current climate change predictions.

5.1 Abstract

Global warming and invasive alien species (IAS) are major contributors to global ecosystems change. For many IAS, it is not currently known whether invaded distributions are in equilibrium with their fundamental ecological niche or how warming may affect future invasion trajectories. This limits the ability of ecosystem managers to anticipate and deal with future IAS expansions. Species Distribution Modelling (SDM) is an effective tool to overcome this as it allows potentially vulnerable areas to be identified and understand how this may change as warming advances. Native to North America, the slipper limpet, *Crepidula fornicata* now ranges in Europe from the Mediterranean Sea to the Irish Sea, and into Norwegian coastal waters, with very large populations in France and the UK. Here, a SDM was used to identify potentially vulnerable areas under current climate conditions, and how climate change may affect this. Based on its current distribution a SDM was developed for *C. fornicata* using occurrence records, and six predictor variables (temperature, salinity, current velocity, chlorophyll concentration, depth, and distance to shore). Distance to shore, temperature, and depth were the variables that contributed most to the model outcome. Areas further north in North America, Europe, and Northeast Asia are most at risk for the introduction of *C. fornicata.*

5.2 Introduction

Global warming, and biological invasions are two of the greatest threats to global biodiversity (Bax *et al*., 2003; Malhi *et al*., 2020). Anthropogenic climate change has resulted in a shift in the phenology and distribution patterns of many species both terrestrial and marine on a worldwide scale (Parmesan *et al*., 1999; Burrows *et al*., 2011; Dambach and Rödder, 2011; Poloczanska *et al*., 2013; Barton *et al*., 2016; Pecl *et al*., 2017). As well as anthropogenic climate change, humans have also resulted in species being introduced outside of their native ranges, both intentionally and unintentionally (Occhipinti-Ambrogi and Savini, 2003). resulting reordering of community structure can affect native ecosystems in a variety of ways and can have direct impacts on the delivery of ecosystem goods and services to human society. The IPCC (Intergovernmental Panel on Climate Change) projects that global average sea surface temperatures (SST) will have increased from 1 °C (Representative Concentration Pathway, RCP 2.6) to over 3 °C (RCP 8.5) by 2081-2100, with warming predicted to be at the highest in the upper 700 m (IPCC, 2014). Previous studies have demonstrated that native and invasive species typically respond in a different manner to environmental stressors, though not always, invasive species show wider ecological tolerances, meaning they could outperform natives when faced with ocean warming (Zerebecki and Sorte, 2011; Sorte *et al*., 2013). It is predicted that temperate species would be more successful when introduced to polar and subpolar ecosystems (Krause-Jensen and Duarte, 2014), than to subtropical and tropical ecosystems. A meta-analysis carried out by Sorte *et al*. (2010) found that 75% of marine range shirts occurred in a poleward direction, and that species shifted an order of magnitude faster in marine environments than terrestrial.

A total of 1517 marine alien species were reported in European seas by the European Alien Species Information Network (EASIN) with Mollusca being the most commonly reported phylum (EASIN, 2021), with only few being invasive. One of these invasive species is *Crepidula fornicata. Crepidula fornicata* is native to the Western Atlantic coast, this gastropod has been introduced repeatedly into Europe during the 19th and 20th Century (Blanchard, 1997), and is now a successful invasive species (Figure 5.1). Its first

introduction occurred accidently in England (McMillan, 1939), then into Europe through the movement of oysters for aquaculture (Thieltges, 2005). The introduction of *Crepidula fornicata* has impacted macro-benthic fauna and flora both on a species level as well as having over impacts on the composition of benthic communities (Thieltges *et al*., 2003). An example of the negative consequence of *Crepidula fornicata* outside of its native range, is that it has been shown to lead to a reduction of survival and growth in the blue mussel *Mytilus edulis* probably as a result of food competition (Thieltges, 2005b). Another effect of *Crepidula fornicata* that has been demonstrated is the resulting increase in siltation due to the accumulation of faeces and pseudofaeces produced by filtration. This has resulted in alterations in benthic sediments and near-bottom currents (Ehrhold *et al*., 1998). The success of *Crepidula fornicata* as an invader appears to be attributable to adults demonstrating extraordinary resilience to many environmental conditions predicted due to climate change (Ries *et al*., 2009; Diederich and Pechenik, 2013; Noisette *et al*., 2016; Kriefall *et al*., 2018).

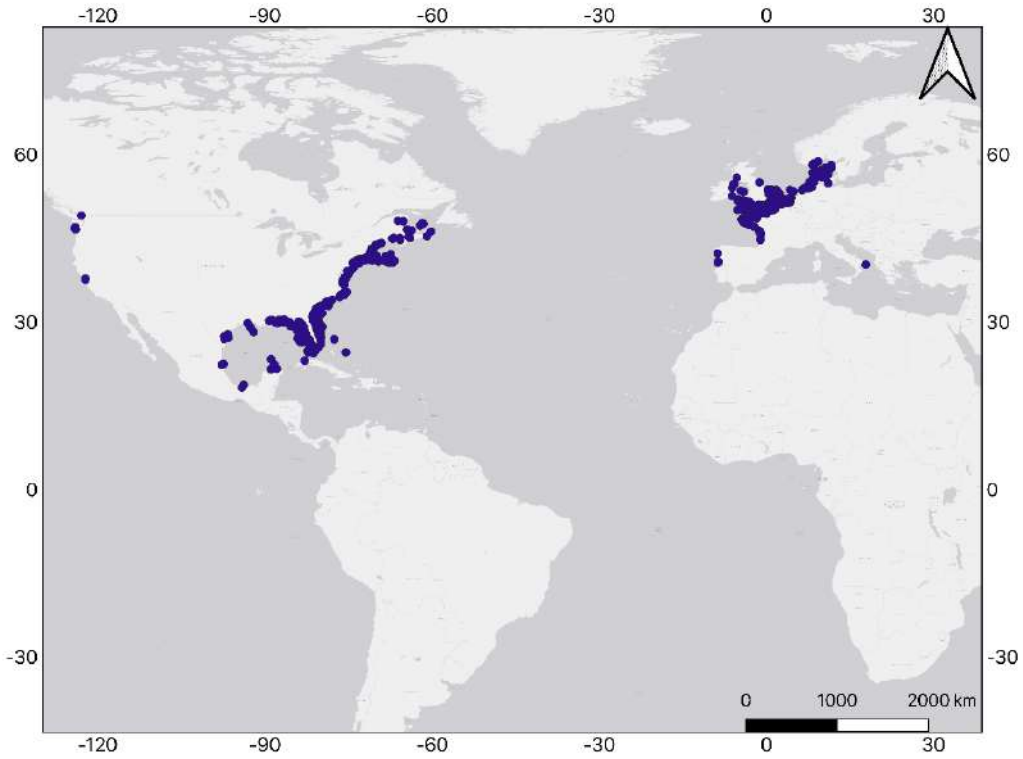

Figure 5.1 Current global distribution of *Crepidula fornicata*

Species Distribution Modelling (SDM) represents one of the most powerful and utilised tools to predict climate driven species redistributions. These models establish a statistical relationship between species occurrence data, and environmental variables, in order to determine distribution patterns, and any changes that may occur with varying environmental conditions (Elith and Leathwick, 2009; Gormley *et al*., 2013). Then by projecting this niche onto future predicted climate conditions an understanding can be gained of areas that may be susceptible to invasion by non-native species. This may allow for ecosystem mangers to anticipate these future shifts, and aim to mitigate against them accordingly (Green and Grosholz, 2021). The management of marine invasive alien species is extremely challenging owing to the high level of connectivity across a broad scale, with eradication only occurring when species have been detected early, and management have responded quickly (Giakoumi *et al*., 2019). This study aims to generate models to identify areas of the world that are vulnerable to invasion by *Crepidula fornicata* but that has yet to occur under current

climate conditions. The study also aims to investigate the potential distribution of *C. fornicata* under two climate change scenarios (RCP 2.6, and RCP 8.5) by 2050 and 2100.

5.3 Material and methods

5.3.1 Geographic distribution

Data on species distribution were gathered from the Global Biodiversity Information Facility (GBIF), and the Ocean Biogeographic Information System (OBIS). Records from GBIF and OBIS were cross referenced to prevent duplication of records. Studies have identified the issue of sampling bias in species occurrence records, and the importance of reducing it. To match with the spatial resolution of marine predictors variables (Table 5.1), only one occurrence per 5 arcminute grid square (approximately 9.2km by 9.2km at the equator) was selected.

5.3.2 Environmental data

In order to develop a species distribution model, it is important to choose environmental variables that are likely to impact the species' distribution (Ready *et al.*, 2010). Environmental variables were chosen based on both on their ecological relevance to *Crepidula fornicata*, along with data availability under present day and future climate scenarios. From Bio-ORACLE, spatial data relating to mean temperature, salinity, current velocity, chlorophyll concentration, and depth was retrieved (Tyberghein *et al.*, 2012). Data on distance to shore was retrieved from the MARSPEC database (Sbrocco and Barber, 2013). The environmental data had a spatial resolution of 5 x 5 arc-minutes. Collinearity between predictor variables can inflate the variance of regression parameters and potentially result in the wrong identification of important predictors in a model (Dormann *et al.*, 2013). Therefore, collinearity of the chosen predictor variables was estimated by calculating the Pearsons correlation coefficients, and ensuring that the value was >-0.7 and <0.7 (Dormann *et al.*, 2013).

Table 5.1: List of environmental variables used to construct the species distribution model of *Crepidula fornicata*

Layer	Unit	Temporal Range	Minimum	Maximum	Mean	Standard deviation
Current Velocity	m/s	2000-2014	1.04×10^{-5}	1.41	0.09	0.09
Depth	m	2016	-10494	2138	-3444	1764
Salinity	PSS	2000-2014	0.06	40.65	34.02	2.38
Sea Surface Temperature	° C	2000-2014	-1.78	30.18	13.82	11.48
Distance to Shore	km	2009	1	2778	680	558
Chlorophyll	mg/m^3	2000-2014	0.016	5.30	0.25	0.19

Table 5.2: Correlation coefficients between the chosen environmental variables

	Current Velocity	Depth	Salinity	Sea Surface Temperature	Distance to Shore	Chlorophyll
Current Velocity	1					
Depth	-0.3	1				
Salinity	0.3	-0.38	1			
Sea Surface Temperature	0.57	-0.28	0.45	1		
Distance to Shore	0.02	-0.47	0.13	-0.08	1	
Chlorophyll	-0.21	0.45	-0.35	-0.56	-0.14	1

Spatial layers with projections of future temperature, salinity, current velocity, and chlorophyll concentrations under two representative concentration pathway emission scenarios (RCP2.6, and RCP8.5) were selected from Bio-ORACLE. Bio-ORACLE provides projections of two future time points, 2040-2050, and 2090-2100. RCP26, is a peak-and-decline scenario resulting in extremely low greenhouse gas concentrations by the end of the 21st century. RCP85 is a more extreme pathway, with increasing emissions over time, causing high greenhouse gas emissions (Moss *et al*., 2010). These models were chosen to represent best and worst case climate change scenarios.

5.3.3 Modelling of species distribution

Maximum entropy (Maxent) models provide a technique for predicting species distributions using only presence data (Elith *et al*., 2011). Maxent is based on the principles of maximum entropy, where a target probability distribution is estimated by finding the probability distribution of maximum entropy, i.e., that is most spread out or closest to uniform, subject to a set of constraints that represent incomplete information about the target distribution (Philips *et al*., 2006). Maxent has been used in areas such as conservation planning, mapping distributions of marine zoonotic

parasites, and invasive-species management (Crafton, 2015; Alt *et al*., 2019; Smith *et al*., 2021). Maxent modelling was implemented using the dismo package (Hijmans *et al*., 2020) for R 4.0.2 (Team, 2020). To evaluate the predictive performance of the model, a cross-validation approach with 10 repetitions was used, the algorithm was trained using 75% of the data and tested using the remaining 25% (Saeedi *et al*., 2017), with 10000 randomly generated background points (Barbet-Massin *et al*., 2012). The predictive performance of the model was evaluated using two metrics: true skill statistics (TSS) (Allouche *et al*., 2006), and area under the receiver operating characteristic curve (AUC) (Fielding and Bell, 1997). TSS values range from -1 to 1, where a value ≤ 0 indicates that the model is giving a random prediction, and a value of 1 indicates perfect model performance (Allouche *et al*., 2006). AUC scores range from 0 to 1, with values < 0.5 indicating the model preforms worse than random, a value of 0.5 indications predictions no better than random discrimination, and 1 indicates perfect discrimination (Gonzalez *et al*., 2011). The most important environmental variables were identified by the contribution rate (represents the importance of a given variable in model training, and permutation importance (Phillips, 2017). The contribution of each factor determined by randomly permuting the values of the factor among the points used for model training and measuring the variation in AUC (training) value. If a large decrease occurs this indicates that the model is heavily reliant on that factor.

For future projections, the model was trained using variables for current conditions and projected using projected mean temperature, projected salinity, projected current velocity, projected chlorophyll, present-day ocean depth, and present-day distance to shore. The future models assume that there will be no major changes in ocean depth, and distance to shore until 2100 (Zhang *et al*., 2019).

5.4 Results

5.4.1 Model performance and variable contribution

The Maxent SDM exhibited high predictive power for *Crepidula fornicata* as evidenced by the high AUC (0.984 ± 0.002), and TSS (0.895 ± 0.005) values (Figure 5.2). The jackknife test (Figure 5.3) results indicate that the distribution of *Crepidula fornicata* was mainly influenced by distance to shore (MS_biogeo05_dist_shore_5m), temperature (BO21_tempmean_ss), and sea depth (BO_bathymean), contributing 70.6%, 21.2%, and 4.2% respectively. Salinity (3.6%) had a minor contribution to predictive performance of the model, whilst chlorophyll concentration (0.2%), and current velocity (0.1%) were mostly irrelevant to the outcome of the model (Table 5.3). With regards to permutation importance, sea depth had the highest impact on the model and contributed 52.6%, followed by sea temperature, contributing 21.2% (Table 5.3). Species response curves illustrate the relationship between environmental variables and the likelihood of species occurrence, they demonstrate biological tolerances for species, and habitat preferences. Response curves of *Crepidula fornicata* (Figure 5.4) to distance to shore, follow that of its current know biology with *C. fornicata* being commonly found in the shallow subtidal up to depths of 60 m. The response curves show that *C. fornicata* are most likely to be found near to the shore (<8 km), and in shallow depths (< 12 m). The model suggests that the most suitable temperature for *C. fornicata* ranges from 13 – 25 °C.

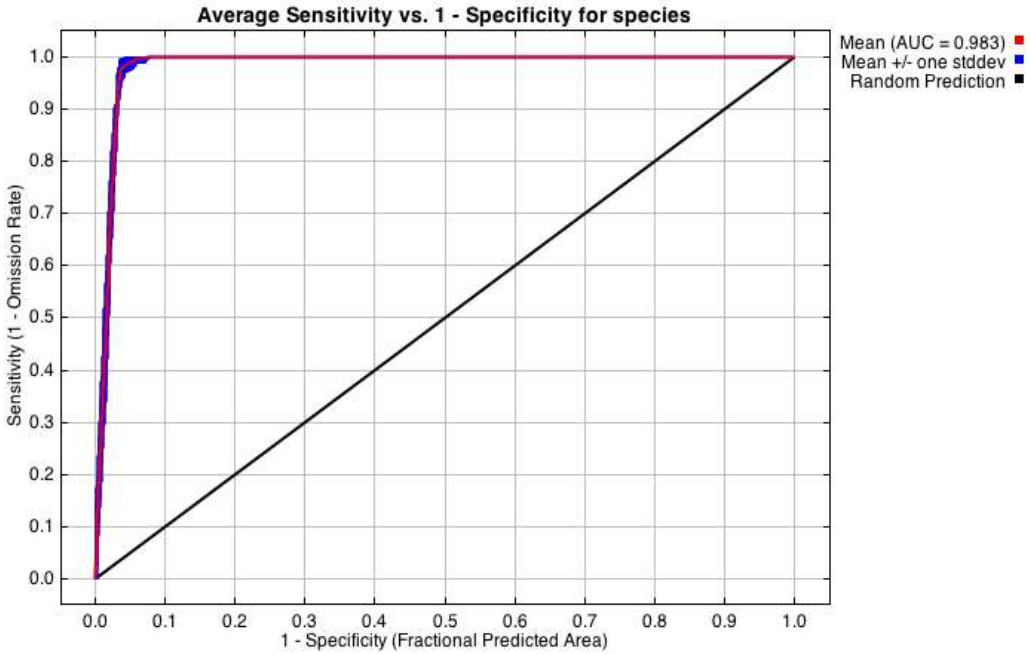

Figure 5.2: ROC curve and AUC value under current climate conditions (10 replicated runs).

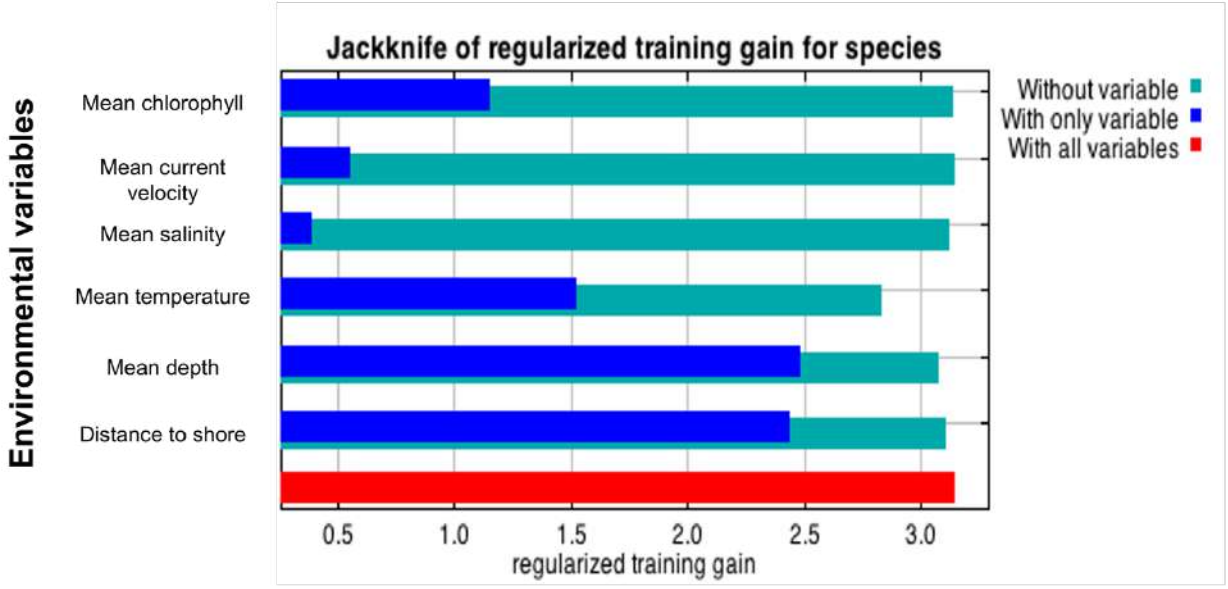

Figure 5.3: Relative predictive power of the chosen environmental variables based on the jacknife of regularized training gain in Maxent models for *Crepidula fornicata*

Table 5.3: Contribution and importance of the six predictor variables used to build the species distribution model for *Crepidula fornicata*

Variable	Contribution (%)	Importance
Distance to shore	70.7	3.5
Temperature	21.2	43.0
Depth	4.2	52.6
Salinity	3.6	0.6
Chlorophyll	0.2	0.1
Current velocity	0.1	0.2

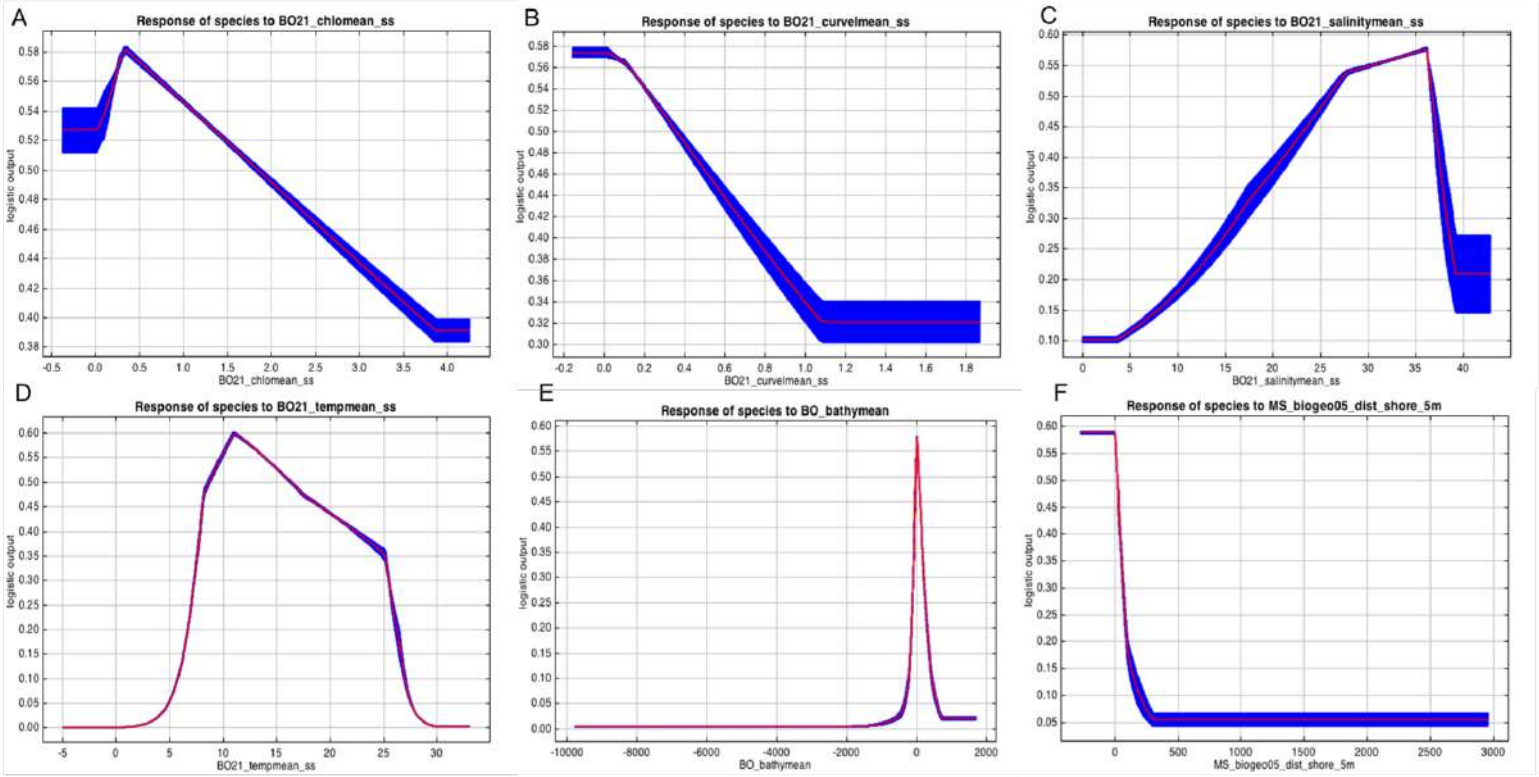

Figure 5.4: Response curves of predicted occurrence probability of *Crepidula fornicata* against (A) Chlorophyll concentration (B) Current Velocity (C) Salinity (D) Temperature (E) Depth and (F) Distance to Shore

Predicted suitable habitat for *Crepidula fornicata* under current climate conditions is shown in Figure 5.5. All occurrence records used for constructing the initial model were within the predicted suitable range. The predictions show that the most suitable environments for *C. fornicata* at present are in the shallow waters of Europe, especially in the Northeast Atlantic waters. Other suitable habitats indicated include areas along the North Pacific coastline, the South Pacific Ocean, the Yellow Sea, and areas of Australia, and some of the New Zealand Coastline. The predictions for *C. fornicata* under future climate change predictions, suggest that there will be an overall increase in the potential suitable habitat for *C. fornicata* (Table 5.4), with an overall increase of 27% possible under the most severe climate change predictions by 2100 (27%). The areas that will see the highest increase in *C. fornicata* are Europe, and North America, with a potential increase of 60%, and 78% respectively under RCP 8.5 predictions by 2100. Within Europe, under the most extreme RCP 8.5 predictions, by 2100, most of Iceland and Norway become a suitable habitat for *C. fornicata.* The majority of southern Europe remains unsuitable, with the exception of Italy (Figure 5.6). In North America under RCP 8.5 by 2100 an increase in suitable habitat is expected further North, in Canadian provinces such as Newfoundland, Ontario, and Quebec, and in Alaska. Areas on the Gulf of Mexico are expected to become less suitable as climate change progresses (Figure 5.7). Under RCP 8.5 predictions, by 2100 North East Asia is expected to experience a 35% increase in habitat suitable for *C. fornicata* mostly attributable to a northward movement into Russia (Figure 5.10). South America is expected to see a slight increase of 6% by 2100 under RCP 8.5 predictions for 2100, with areas in the South becoming more suitable such as Chile, and the Falkland Islands (Figure 5.8). Areas further north such as Brazil are predicted to be less suitable for *C. fornicata*. Some areas are expected to see an overall decline in the suitable habitat for *C. fornicata*, with Australia and New Zealand experiencing a 30% decline, and Africa experiencing a 30% decline. Under less severe climate predictions for 2050, Europe and North America are still expected to experience a large increase in the potential suitable habitat for *C. fornicata*, of 46% and 15% respectively. Australia and New Zealand, Asia, and Africa are all likely to experience a decrease in suitable habitat for *C. fornicata* under the less severe RCP 2.5 predictions for 2050.

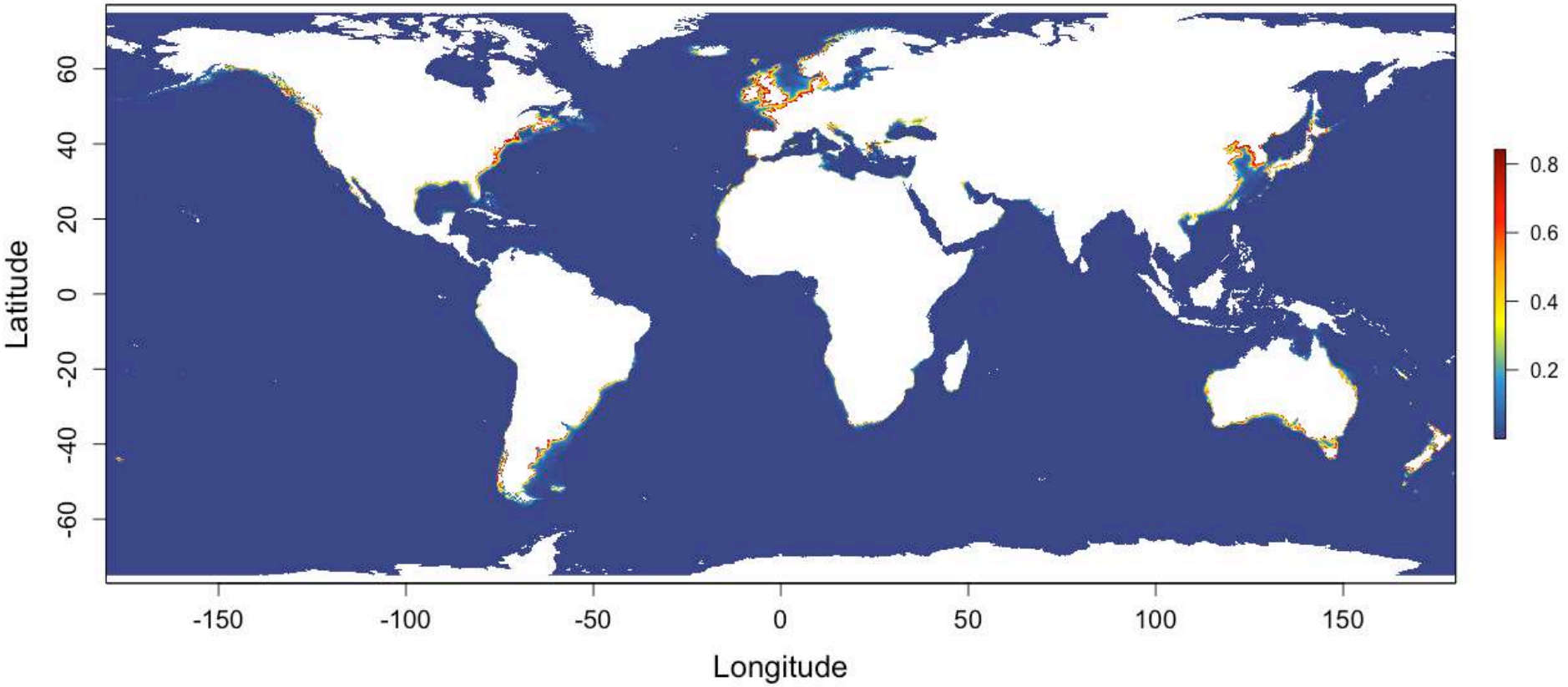

Figure 5.5: Potential global distribution of *Crepidula fornicata* under current climate conditions

5.4.2 Potential Future Distribution

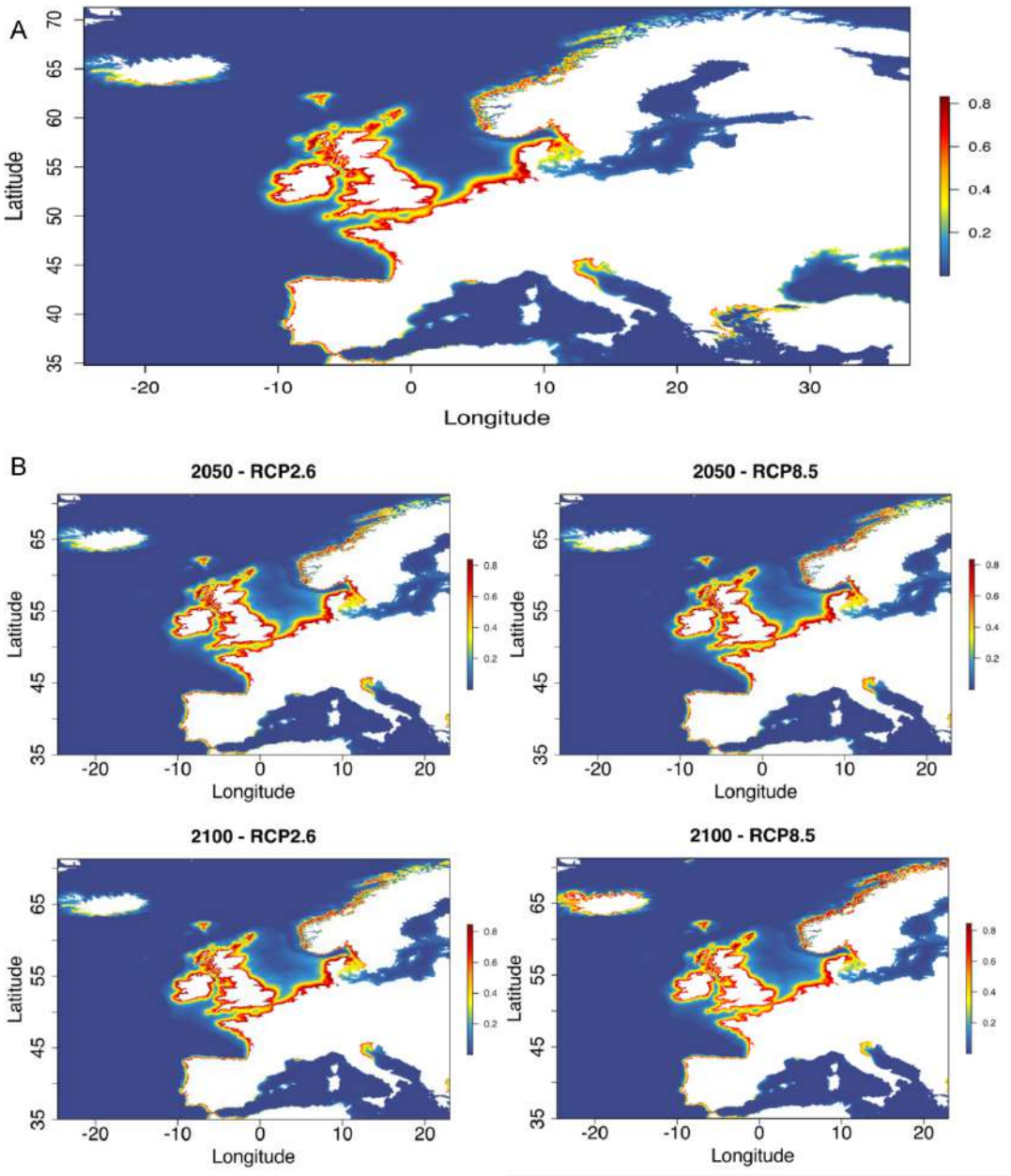

Figure 5.6: (A) Potential distribution of *Crepidula fornicata* within Europe under current climate conditions. (B) Predicted range shift of *C. fornicata* in Europe under RCP 2.6, and RCP 8.5 from the period 2050 and 2100.

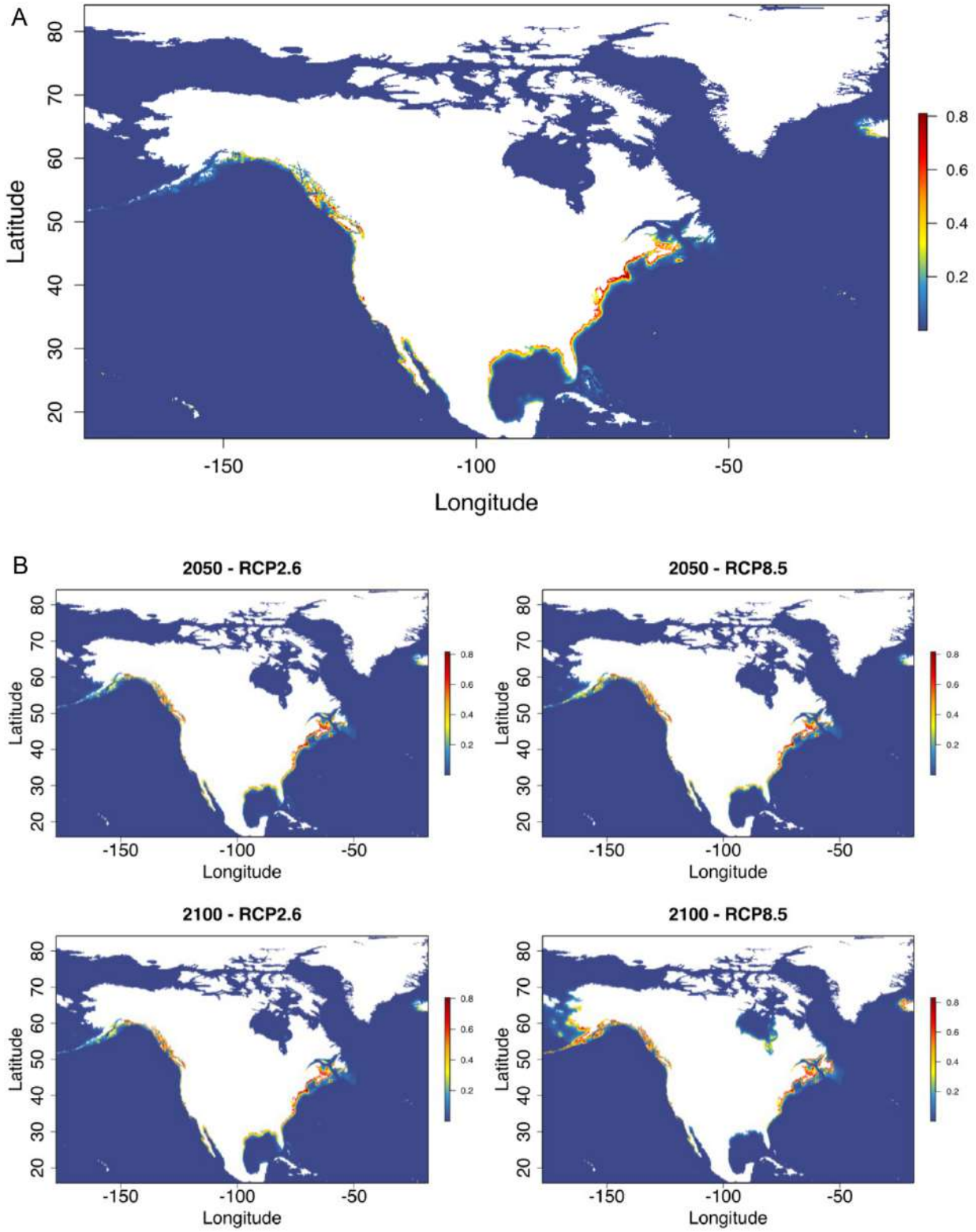

Figure 5.7: (A) Potential distribution of *Crepidula fornicata* within North America under current climate conditions. (B) Predicted range shift of *C. fornicata* in North America under RCP 2.6 and RCP 8.5 during 2050 and 2100.

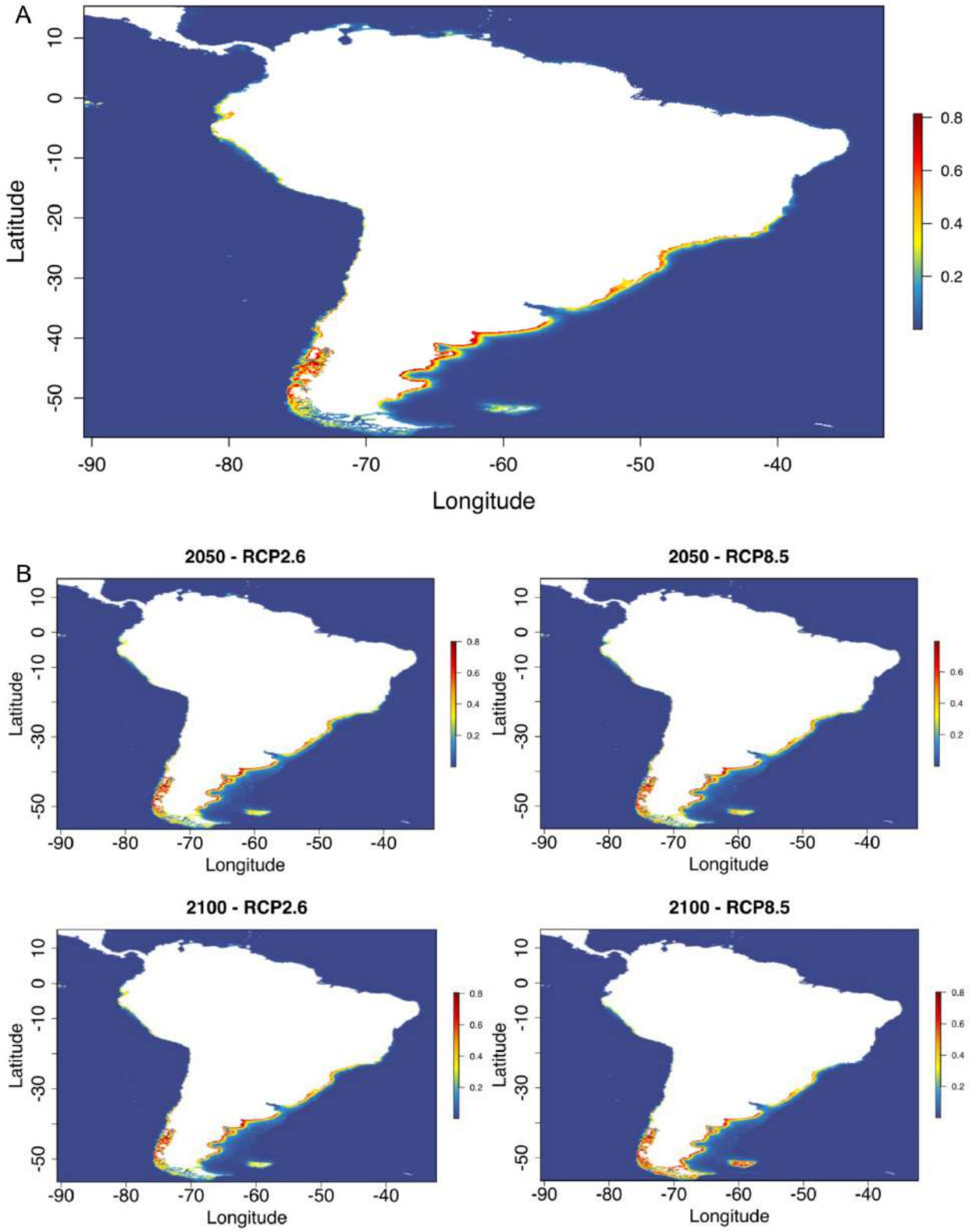

Figure 5.8: (A) Potential distribution of *Crepidula fornicata* within South America under current climate conditions. (B) Predicted range shift of *C. fornicata* in South America under RCP 2.6 and RCP 8.5 during 2050 and 2100.

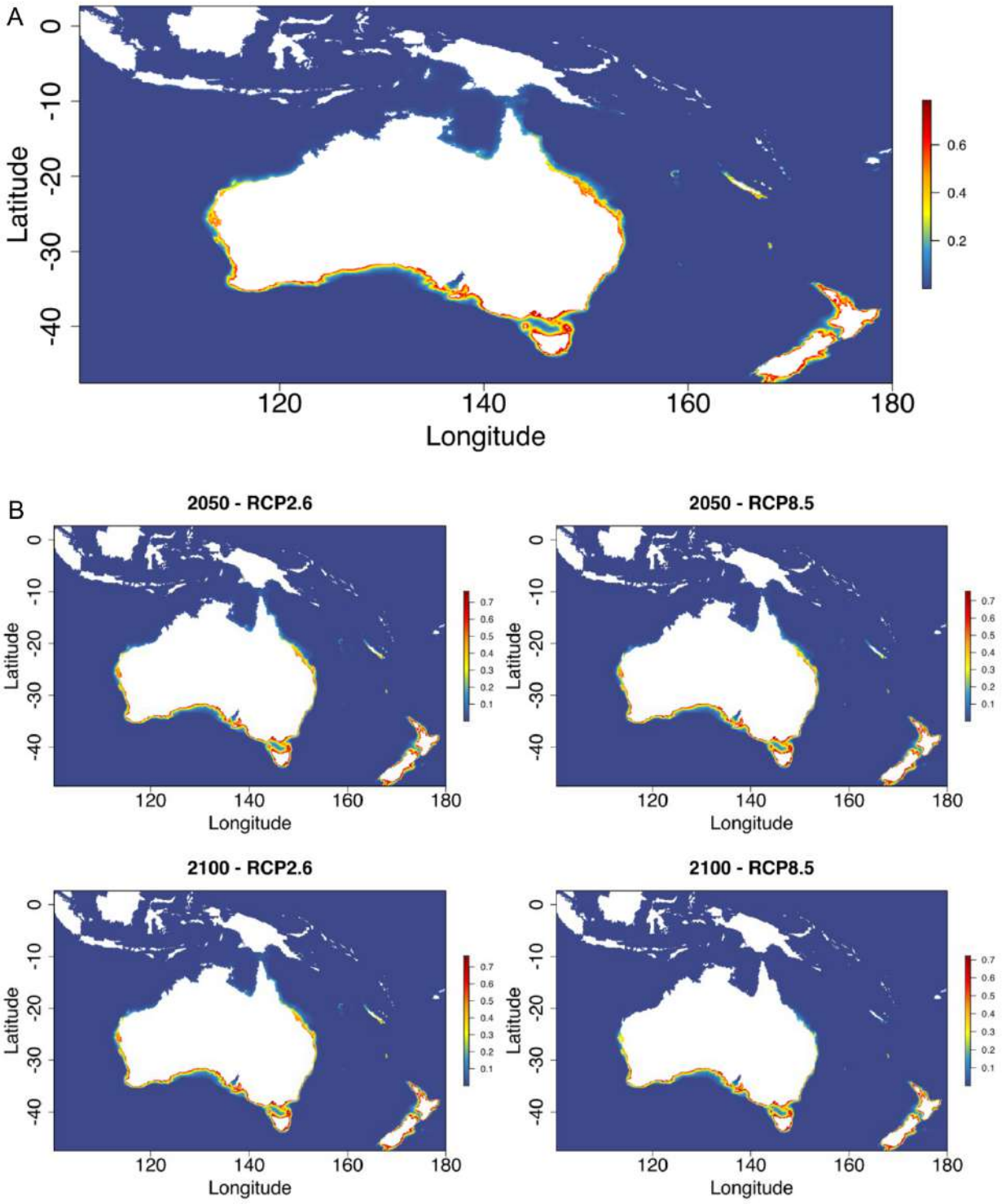

Figure 5.9: (A) Potential distribution of *Crepidula fornicata* in Australia and New Zealand under current climate conditions. (B) Predicted range shift of *C. fornicata* in Australia and New Zealand under RCP 2.6 and RCP 8.5 during 2050 and 2100.

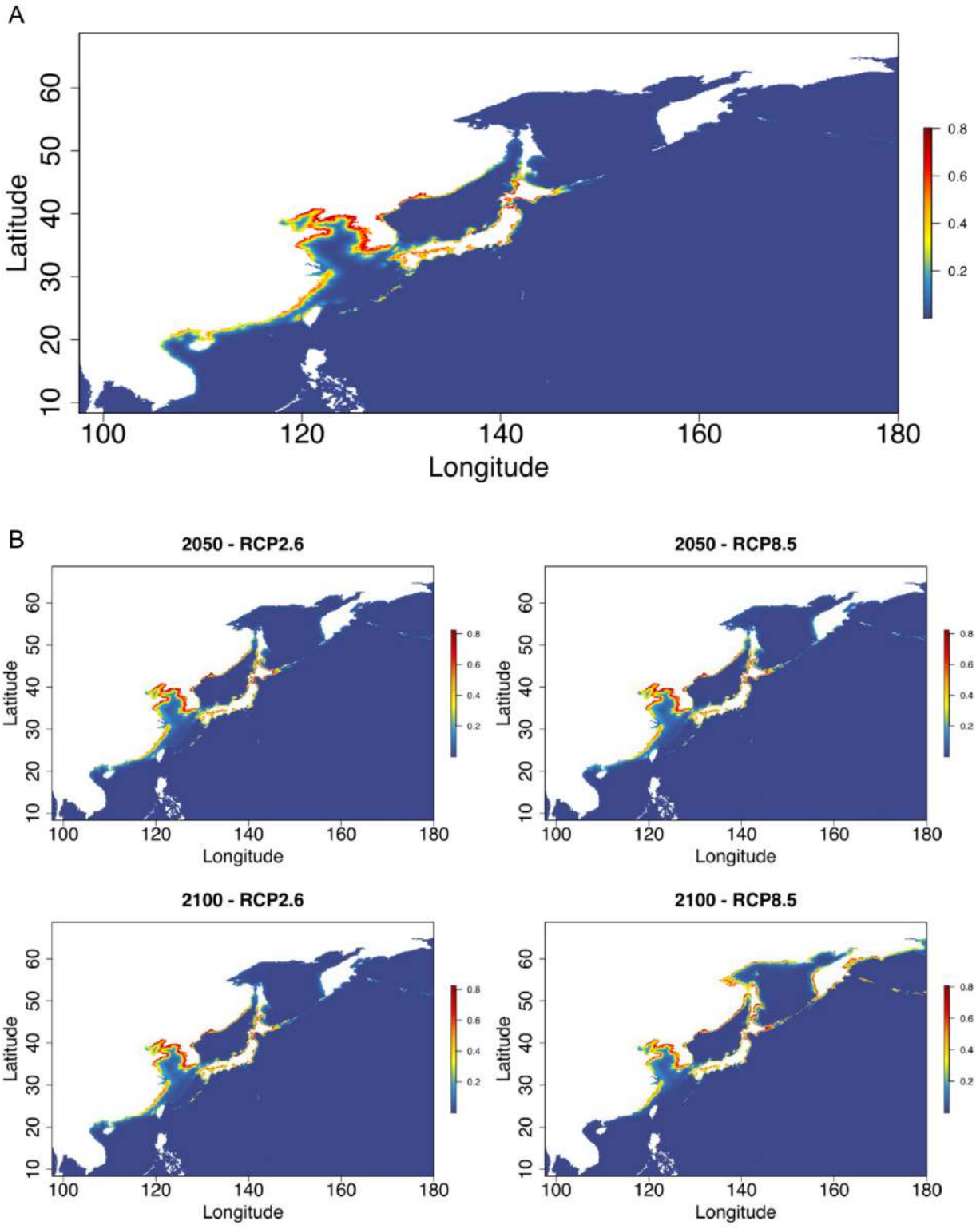

Figure 5.10: (A) Potential distribution of *Crepidula fornicata* in East Asia under current climate conditions. (B) Predicted range shift of *C. fornicata* in East Asia under RCP 2.6 and RCP 8.5 during 2050 and 2100.

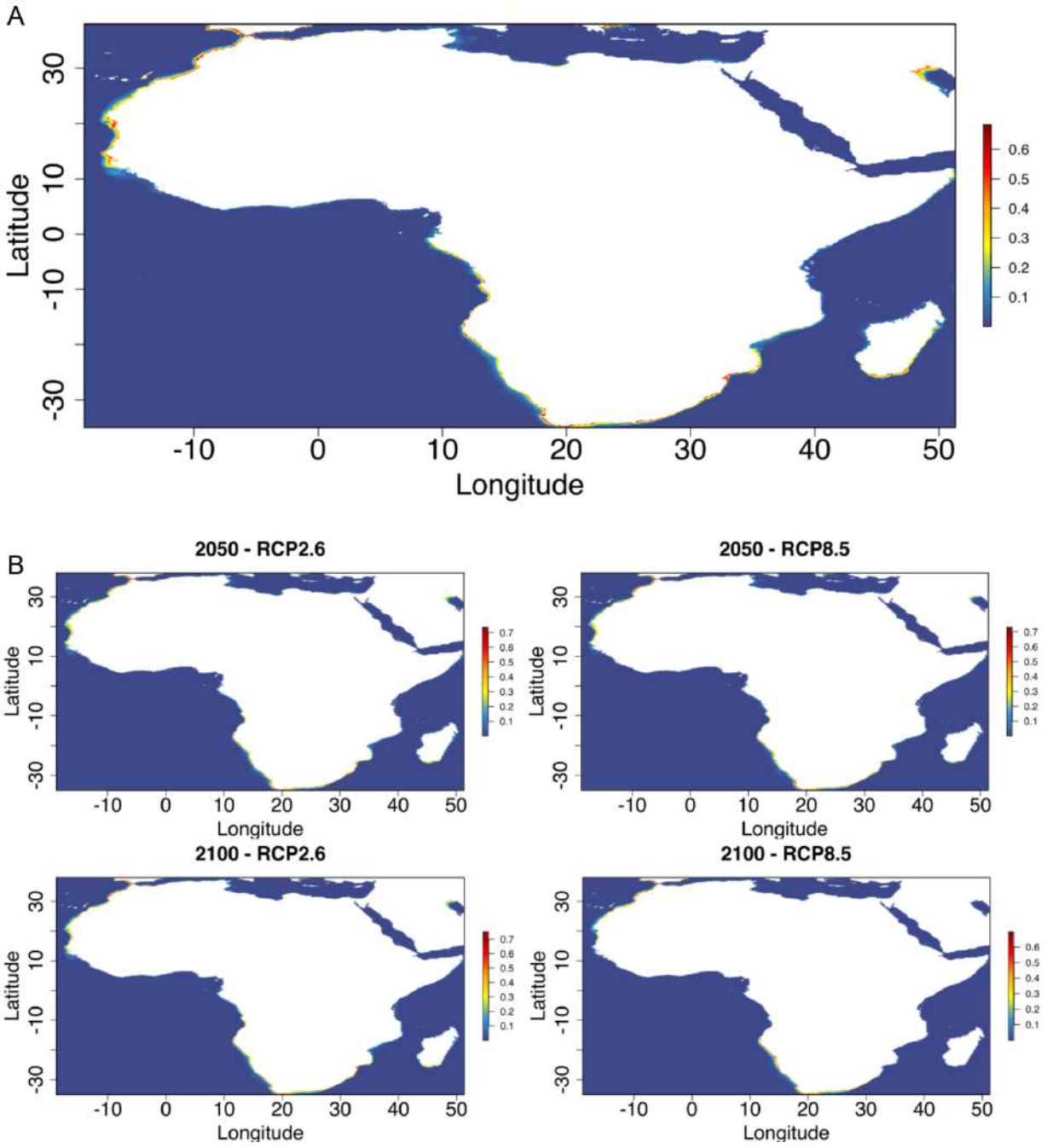

Figure 5.11: (A) Potential distribution of *Crepidula fornicata* in Africa under current climate conditions (B) Predicted range shift of *C. fornicata* in Africa under RCP 2.6 and RCP 8.5 during 2050 and 2100.

Table 5.4: Change in suitable habitat (%) of *Crepidula fornicata* under IPCC RCP 2.6, and RCP 8.5 predictions for 2050 and 2100.

	2050 - RCP 2.5	2050 - RCP 8.5	2100 -RCP 2.5	2100 – RCP 8.5
Overall	2%	2%	2%	27%
Europe	46%	48%	49%	61%
North America	15 %	18%	14%	78%
South America	3%	3%	-0.1%	6 %
Australia/New Zealand	-10%	-15%	-9%	-30%
Asia	-1%	1%	0.07%	35%
Africa	-18%	-24%	-15%	-45%

5.5 Discussion

Range shifts as a response to climate change have been reported in a number of species both terrestrial and marine (Parmesan *et al*., 1999; Perry *et al*., 2005; Chen *et al*., 2011; Lenoir *et al*., 2019). In this study, a Maxent model for *C. fornicata was* developed to investigate the effects of climate change on its potential global distribution. The Maxent SDM showed good predictive abilities and indicated that *C. fornicata* has not yet occupied the full extent of its present-day potential suitable habitats worldwide and warming will facilitate its expansion over the coming decades.

Among the predictor variables chosen to build the SDM, distance to shore, temperature, and depth were the three variables that influenced the distribution of *C. fornicata*. As *Crepidula fornicata* is most commonly found in the intertidal zone down to depths of 60 m (Blanchard, 1997), it is not surprising that these variables play a critical role in regulating their distribution. According to the SDM, under present-day climate conditions, the potential range of *C. fornicata* is larger than its current distribution. Areas that could potentially be suitable for *C. fornicata* include areas along the North Pacific coastline, the South Pacific Ocean, the Yellow Sea, and areas of Australia, and much of the New Zealand coastline. Therefore, it is important to ensure that human mediated activities such as shipping and aquaculture do not allow for the movement of *C. fornicata* into these uncolonized areas. It is possible that the result of a larger potential range than the current known distribution is due to the lack of knowledge of factors that are impeding the establishment of *C. fornicata* into these areas.

Future model projections from 2050 and 2100 obtained using IPCC climate change scenarios indicate that climate change will impact the distribution of *C. fornicata,* but the impacts will differ from location to location. Generally, in response to climate change, the model predicts that areas suitable for *C. fornicata* will expand in many areas but there are also areas where range contraction will take place. In Europe, North America, and Northeast Asia under RCP8.5 concentration pathways a significant

northwards expansion is predicted. Range expansions by species can act as a threat to marine ecosystems by modifying species interactions, and to humans dependent on marine resources. For example, as a result of increased poleward infiltration of the warm East Australian Current, the sea urchin *Centrostephanus rodgersii* (Agassiz, 1864), has undergone a southern range expansion in temperate southeastern Australia (Edgar et al., 2004, 2005; Johnson et al., 2005; Ling et al., 2008). *Centrostephanus rodgersii* has extended from New South Wales (NSW) south to the Tasmanian coastline (Johnson *et al*., 2011; Strain *et al*., 2013). Strain *et al*. 2013 found that the introduction of urchins to algal beds resulted in abalone fleeing and seeking shelter in cryptic microhabitats, potentially negatively impacting the productivity of abalone fisheries.

It is important to note that the RCP 8.5 concentration pathway is considered a "worst-case scenario", and if there were to be a decrease in greenhouse gas emissions, then ocean warming would happen at a reduced rate (IPCC, 2019). Though there have been some who have characterized RCP 8.5 as "extreme", and "misleading" (Peters and Hausfather, 2020), the emissions consistent with RCP 8.5 are in close agreement with historical total cumulative CO_2 emissions, and is also the best match to 2050 under current and stated policies, but with still be extremely plausible CO_2 emission levels by 2100 (Schwalm *et al*., 2020). As all IPCC scenarios contain a degree of uncertainty, it is important for policymakers to consider all possibilities including "worst-case scenarios".

The SDM in this study was developed using six abiotic predictors, however it is possible that factors outside of the chosen six could also be important in determining the distribution of *C. fornicata.* Bohn *et al.* (2015), determined that *C. fornicata* in Welsh coastal waters was not entirely limited by the environmental conditions in the area, but that other factors may be contributing to the lack of northward expansion. All factors that limit *C. fornicata* dispersal besides climate conditions are not yet fully understood, but factors such as the availability of settlement substrata may be a contribution factor (Bohn *et al*., 2015). Lynch *et al*. (2020) also found that nearshore meteorological extremes can impact the success of the Mediterranean mussel *Mytilus galloprovincialis* in Ireland, despite future climate change scenarios predicting its northward establishment.

Crepidula fornicata has many traits that could enable it to succeed despite future climate change conditions. Diederich and Pechenik (2013) studied the thermal tolerance of *C. fornicata* at different life stages from both intertidal and subtidal populations. They found that *C. fornicata* had a high thermal tolerance with the lethal range being 33-37°C following a 3 h exposure. They also found embryos were more tolerant than adults to higher temperatures. This is unusual as early life stages tend to be more sensitive that adults to thermal stress (Truebano *et al*., 2018; Pandori and Sorte, 2019), Diederich and Pechenik (2013) suggest that intertidal *C. fornicata* may be living close to their upper thermal limits, when they studied populations in their native range of Rhode Island, USA, and will most likely move to subtidal areas as temperatures continue to rise. Kriefall *et al*. (2018) studied the effects of ocean acidification on *C. fornicata* and found that in the presence of severe ocean acidification stress, that the larvae of *C. fornicata* showed some negative but overall mild effects, especially when compared to that of other marine species. A study also found that rising seawater temperatures in northern European waters could increase the reproductive output of *C. fornicata,* both as a result of improved gametogenic/brooding temperatures as well as increased phytoplankton availability (Valdizan *et al*., 2011).

Generally, the eradication of an established invasive species is incredibly difficult, and often only successful if the species has been detected early, and management has responded quickly (Giakoumi *et al*., 2019). Even for known highly invasive species such as *C. fornicata,* regular monitoring to detect its presence in at risk areas is not in practice, recently eDNA markers to easily detect *C. fornicata* have been developed (Miralles *et al*., 2019). The use of eDNA markers is one option to monitor the presence of *C. fornicata* in areas where it is yet to be established to ideally manage any future invasions. It could be useful for future studies to combine SDMs with particle tracking to further enhance the understanding of the distribution of invasive species such as C. fornicata and to predict functional connectivity (Wang *et al*., 2020). Particle tracking can combine physical advection and diffusion with biological development and behaviour to allow particles to move through defined developmental stages (Wood *et al*., 2021).

5.6 Conclusion

The maximum entropy model using six environmental variables was used to evaluate the potential suitable habitat distribution of *C. fornicata* both under current and future climate projections using two IPCC scenarios. It was found that *C. fornicata* is likely to increase in distribution, and in Europe, North America, and Northeast Asia, move northwards under the most severe climate change predictions. *Crepidula fornicata* if allowed to enter a new area is likely to succeed and outcompete many native species due to range of factors such as its resilience to ocean acidification and warming temperatures favouring larval growth (Bashevkin and Pechenik, 2015).

Chapter 6 General Discussion

At the outset, the main aims of this thesis were to firstly address the lack of knowledge with regards to the haemolymph biochemistry of *Crepidula fornicata*. A focus was then placed on the disease harbouring potential of *Crepidula fornicata* especially those diseases known to be potentially detrimental to shellfish and/or human health. When an invasive species becomes introduced to a new area there are many potential outcomes such as the introduction of new diseases, becoming accidental hosts for pathogens already present in their new environment or possibly facilitating a native species by reducing parasite prevalence by means of a "dilution effect" (Crowl *et al*., 2008; Geburzi and McCarthy, 2018; Westby *et al*., 2019). A final objective of this thesis was the construction of a Species Distribution Model in order to map the potential geographical distribution of *C. fornicata* under present and future climate change scenarios.

Chapter 1 (Introductory Literature Review) highlighted that much of the current efforts to understand the health status of molluscs is restricted to some key commercially important species e.g., *Mytilus edulis,* and *Haliotis* spp. A distinct lack of literature could be found with regards to numerous aspects of the invasive alien species *C. fornicata,* namely its health status and physiology. To fully understand the potential impacts of an invasive alien species, many factors need to be considered. Although substantial research efforts have gone into better understanding the general ecology and impacts of *C. fornicata* outside of its native range, few studies have been performed to determine their disease harbouring potential, or to understand various aspects of their haemolymph biochemistry. Chapter 1 highlighted that currently little to no concerning and/or notifiable diseases have been described in *C. fornicata.* However, it is difficult to ascertain if this truly was the case given the lack of literature available. This body of work presents a series of studies designed to address these knowledge gaps and to gain an understanding of the potential of *C. fornicata* to act as a source/sink of infectious diseases that could be potentially detrimental to commercially important, co-located shellfish. This study also aided in increasing the understanding of the haemolymph biochemistry of *C. fornicata* through the characterisation of phenoloxidase-like activity.

Chapter 2 explored factors associated with the immunobiology of *Crepidula fornicata.* Results showed that using a range of specific and non-specific substrates, laccase and catecholxidases based enzymatic activity was detected for the first-time in the haemolymph of *C. fornicata.* The by-products were further studied and found to have broad-spectrum antibacterial properties. It is possible that this broad-spectrum antibacterial property of phenoloxidase goes part ways to explaining the lack of disease signatures present in *C. fornicata* that were PCR positive for the Vibrio-like bacteria. Melanin was also visualised across a variety of tissue types. Phenoloxidase activity and melanization is a hallmark of most invertebrate innate immunity, both terrestrial and aquatic (Tang, 2009; Mydlarz and Palmer, 2011; Palmer *et al*., 2011; Whitten and Coates, 2017; Tassanakajon *et al*., 2018). From the data, it is clear that at least two types of PO-like activity i.e. laccase and catecholoxidase contributes to the biological defence mechanisms in *C. fornicata.* This study is an important starting point to understand the immune defences of *C. fornicata*, an area which is currently without knowledge. As bacterial defenses of PO can be affected by other immune factors including lysozyme, superoxide dismutase etc, it would be interesting to further investigate PO interactions with other factors of bacterial resistance (Wootton *et al*., 2003; Pruzzo *et al*., 2005;). As climate change will likely cause shifts in multiple environmental factors simultaneously e.g. temperature and salinity, it could be interesting to study how this combined stress might alter PO activity in *C. fornicata,* and therefore their future susceptibility and/or resistance to episodes of infection.

Chapters 3 and 4 attempt to close the knowledge gap by looking systemically across two affected sites in Wales, focussing on *Vibrio* in the first instance (a major disease pathogen), and several others known to impact molluscs globally (Haplosporidians/microsporidians/paramyxids). A multi-resource screen is used in addition to looking at the population dynamics across an oyster restoration zone (Swansea Bay), and an area of diverse commercial activity (Milford Haven).

Chapter 3 investigated the potential role of *Crepidula fornicata* as a sink for Vibrio-like bacteria, at two study sites in South Wales, Swansea Bay and Milford Haven. Vibriosis,

caused by certain *Vibrio* species, is one of the most prevalent bacterial disease affecting hatchery bivalve production (Dubert *et al*., 2017). Results showed an extremely high prevalence of *Vibrio* bacteria among the sampled *C. fornicata*. However, *C. fornicata* at the two sites surveyed were not susceptible to *Vibrio* bacteriosis. Importantly, the *C. fornicata* at both study sites were not found to be acting as a reservoir for any notifiable *Vibrio* species for human-related food poisoning/gastroenteritis. For example, the notable pathogens of molluscs, *V. parahaemolyticus* and *V. aestuarensis* were not found in the haemolymph of *C. fornicata* implying that other resident (native) bivalve and gastropod molluscs are not affected by the presence of this invader. Challenge trials could be carried out on *Crepidula fornicata* to experimentally investigate infection parameters of these key pathogens i.e. *Vibrio parahaemolyticus* and *V. aestuarensis.* It would be insightful to determine the level of infection required to begin to notice the effects in *Crepidula fornicata.* Travers *et al*., 2017 studied oyster infection dynamincs by *Vibrio aestuarianus* by conducting experiments to compare miminal infective dose, lethal does 50 and bacterial shedding for six strains of *V. aestuarianus.* Furthermore, the presence of *C. fornicata* does not appear to pose a risk to human health by harbouring pathogenic *Vibrio*. In countries such as France, the U.S.A, Spain, and Morocco, efforts are being made to market *C. fornicata* as a food product (Fitzgerald, 2007). A seasonal effect was noted, with the warmer summer months having a higher prevalence of Vibrio-like bacteria. This has been noted in a number of other studies (Yang *et al*., 2021). This link between warmer weather and increased *Vibrio* prevalence is an important factor to note when considering the potential effects of predicted ocean warming (Genner *et al*., 2017). Future ocean warming will increase the prevalence of Vibrio-like bacteria.

Data collected in Chapter 4 overwhelmingly suggest that *C. fornicata* at both study sites in Milford Haven and Swansea Bay do not appear to be acting as a reservoir for any notifiable pathogens of either shellfish and/or humans such as haplosporidians, microsporidians, or paramyxids. However, in a small number of slipper limpets both turbellarians, and trematodes were identified through whole-tissue histology. Invasive alien species can affect parasite transmission in one of three ways: the first by

introducing non-endemic parasites, i.e "spillover", secondly by successfully transmitting endemic parasites resulting in an overall increased transmission, i.e. "spillback", or finally by transmitting endemic parasites but with a lower transmission potential, i.e. a "dilution" effect (Kelly *et al*., 2009; Johnson and Thieltges, 2010; Mitchell and Leung, 2016). Due to the lack of available literature on the disease status of *C. fornicata* in its native range, it is impossible to state that *C. fornicata* has not transferred pathogens or parasites from its native range to its invaded area. The data collected in this thesis suggest that *C. fornicata* may have the potential to act as a spillback host. Some individuals were found to be infected with a trematode, which has since been identified to be a microphallid parasite, a putative novel species related to the genus *Longiductotrema* (Thomas, Coates and Rowley, unpublished observations). These microphallids are parasites of birds and fish, with crabs and other crustaceans as second intermediate hosts. Given its low prevalence (*ca.* 3%) it is likely that *C. fornicata* has become an accidental host in the two study locations and that the principal primary intermediate hosts are probably various native littorinids. Despite shellfish, such as crabs, in the locality being known to be infected with pathogens such as haplosporidians, and microsporidians (Davies et al., 2020, 2021), *C. fornicata* appears to lack general vulnerability to these either as main or accidental hosts. This ability to not succumb to disease could be an important reason for its success as an invader. The unusual cells reported in Chapter 4 in the epithelial wall of the slipper limpet's GI tract may be virally-infected cells. A recent study of novel viral infections in crustaceans showed cells in the hepatopancreas that closely resemble those in the current work with distorted and marginalised chromatin and eosinophilic cytoplasm (Bateman *et al*., 2021).

Finally, Chapter 4 also highlighted the importance of utilising a multi-resource approach (e.g., a combination of PCR and histology) in determining the disease status of an animal, so as to avoid any issues regarding false positives. Quite often, PCR alone is utilised to detect the presence or absence of specific disease-causing agents. PCR results in chapter 4 indicated *Crepidula fornicata* individuals to be positive for the presence of haplosporidians, microsporidians and paramyxids, however Sanger sequencing

confirmed these results to be false positives. These Sanger sequencing results were also in line with histology results that indicated no signs of infections in these PCR-positive individuals. Though PCR is a robust tool for the detection and characterization of diseases (Lynch et al., 2005, 2013a; Correa et al., 2006; Schrader et al., 2012; Albuixech-Martí et al., 2020; Bao et al., 2021), the issue of false positives arising has been noted in several studies (Bryson, 2014; Hitakarun *et al*., 2014; Jaroenlak *et al*., 2016; Canier *et al*., 2020). Claydon *et al*. (2004) found that the primers initially used to detect the notifiable crustacean pathogen white spot syndrome virus (WSSV) in the Australian red clay crayfish *Cherax quadrincarinatus* (Von martens, 1868) produced false positive results. Claydon *et al*. (2004) determined this to be caused by a lack of sufficient primer specificity for WSSV in the original test, and suggests that PCR results be confirmed with a different method e.g. sequencing. As some of the disease targets screened for in Chapter 4 could potentially be notifiable diseases e.g. the *Hapolosporidia* protozoan parasite *Bonamia ostreae* (OIE, 2019), it proved essential to confirm any PCR results through other means, i.e. Sanger sequencing and histology. The misdiagnosis of a notifiable disease can have massive effects, e.g. the misdiagnosis of WSSV at an Australian aquaculture centre resulted in the immediate destruction of all crustaceans at the site (Claydon *et al*., 2004).

Invasive species, can disrupt local infection dynamics also in an indirect manner, i.e. not acting as pathogens hosts but through competitive and trophic interactions with native species or through the modification of local habitats, thereby altering the abundance and/or contact rates amongst native host species, parasite infective stages, or vectors (Chinchio *et al*., 2020). There are many literature sources available on the ecological impacts of *C. fornicata* outside of its native range (Thieltges, 2005a; Thieltges *et al*., 2006b) with this thesis going some way to address the paucity of knowledge regarding *C. fornicata* epizootiology and their impact on local infection dynamics.

Chapter 5 demonstrated the potential future spread of *C. fornicata* because of climate change. Areas further north in North America, Europe, and Northeast Asia appear to at the most risk for the introduction of *C. fornicata*. Climate-induced changes can affect the

health and productivity of marine ecosystems. Many pathogens of marine organisms are sensitive to factors such as temperature and rainfall (Esteves *et al*., 2015; Joint and Smale, 2017; van Gestel *et al*., 2020), all of which can be affected by climate change. Marine parasites show optimum temperatures for successful life-cycle completion, and if this season extends then there is the potential for increased proliferation of parasites to occur, and a longer time for host infection (Barber *et al*., 2016). The impacts of temperature on trematodes has been well studied, and it was found that increased environmental temperatures are typically associated with a large increase in the output of cercariae into the external environment, as high temperatures accelerate both the production of cercariae within developing rediae and trigger their emergence (Ataev, 1991). Climate induced warming can increase host susceptibility but it can also increase pathogen development and survival rates, thereby increasing disease incidence (Tracy *et al*., 2019). The introduction of marine species to a new area, due to changing environmental conditions, can allow for the introduction of novel diseases. Though currently in the study sites chosen, *C. fornicata* does not appear to have transported diseases from its native range to its invaded range, it's predicted future expansion due to climate change, means that it remains a species that will need to be monitored. Given the lack of parasites/pathogens present in *C. fornicata* their spread may be entirely dependent on abiotic/environmental barriers, unless they move into an area with parasites that they have not previously encountered. It is also likely that *C. fornicata* will continue to impact any newly invaded areas as it currently does, through issues such as modifications to trophic structures, and changes in sediments and near-bottom currents. Plans to restore native oysters, *Ostrea edulis*, into such areas would seem to be futile as slipper limpets out compete these for space and resources both now and in the future.

In conclusion, it would appear currently that the presence of *C. fornicata* in local shellfish grounds in South Wales is not a concern with regards to them acting as a reservoir of potentially harmful diseases, though some may still regard them as a general pest due to their high numbers in the area. It remains important, however, to

continue to monitor their health status, especially with regards to the potential impacts of climate change on disease prevalence and severity.

6.1 Future Research

An important study that needs to be carried out is an extensive disease screen of *Crepidula fornicata* in its native range. This would aid in understanding the diseases *Crepidula fornicata* could potentially be transporting outside of its native range. To date, little disease screen work has been carried out in its native range, with a screen for the presence of trematodes in *C. fornicata* in Rhode Island, USA by Pechenik *et al*., (2001) being one of the only.

A future step from the research described in this thesis would be to expand on the disease targets chosen. An obvious area would be that of viruses. Viruses are the most abundant entities in the oceans, with an abundance estimated to be up to 10^8 mL^{-1} (Welsh *et al*., 2020). Much of the knowledge regarding these is restricted to farmed species such as oysters *Crassostrea gigas*, abalone *Haliotis diversicolor supertexta* and the scallop *Chlamys ferreri* (see Arzul *et al*. 2017). Shellfish can act as hosts for viruses both detrimental to other shellfish but also to humans. Key shellfish viruses include *Ostreid herpesvirus*, which has resulted in large scale die-offs in oyster farms globally (Lynch *et al*., 2012; Bai *et al*., 2015; Gutierrez *et al*., 2020). Shellfish can also carry viruses detrimental to human health e.g. norovirus (Gyawali *et al*., 2019), and more recently Hepatitis E Virus (La Bella *et al*., 2021; Mateus *et al*., 2021).

From the outcomes of the Species Distribution Modelling, further research combining SDMs with particle tracking to further expand the understanding of the distribution of *C. fornicata* and to predict functional connectivity could be employed. To prevent the establishment of an invasive species, it is necessary to respond quickly. If increasingly accurate predictions can be made, along with the use of eDNA monitoring, then it may

be possible to prevent the establishment of an invasive non- native species in a new area.

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Appendix

Declaration:

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Paper 1: Laccase and catecholoxidase activities contribute to innate immunity in slipper limpets, *Crepidula fornicata*.

Located in Chapter: Chapter 2

Author 1: Emma Quinn, Swansea University. Lead author, carried out experiments and data handling.

Author 2: Mrs Sophie Malkin, Swansea University. Assisted with histology

Author 3: Professor Andrew Rowley, Swansea University. Provided training for histology, provided histology analysis.

Author 4: Dr Christopher Coates, Swansea University. Primary investigator: conceived the study, data analysis and figure preparation, drafted the manuscript, revised the manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published peer-reviewed manuscripts contributing to this thesis:

Author 1: Emma Quinn 15/09/21

Author 2: Sophie Malkin 15/0/21

Author 3: Andrew Rowley 15/09/21

Author 4: Christopher Coates 15/09/21

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Laccase and catecholoxidase activities contribute to innate immunity in slipper limpets, *Crepidula fornicata*

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ABSTRACT

The slipper limpet *Crepidula fornicata* is an invasive, non-native, marine species found throughout the coastal waters of southern England and Wales, UK. These limpets are considered to blight commercial shellfish banks, notably oysters, yet little is known about their disease-carrying capacity or their immunobiology. To address the latter, we isolated haemolymph (blood) from limpets and tested for the presence of the immune-enzyme phenoloxidase. Invertebrate phenoloxidases produce melanic polymers from simple phenolic substrates, which are deployed in the presence of pathogens because of their potent microbicidal and microbiostatic properties. We used a series of established substrates (e.g., tyrosine, hydroquinone) and inhibitors (e.g., 4-hexylresorcinol, benzoic acid) to target three distinct enzymes: laccase (*para*-diphenoloxidase), catecholoxidase (*ortho*-diphenoloxidase) and tyrosinase (monophenoloxidase). We confirmed laccase and catecholoxidase activities and characterised their kinetic properties across temperature and pH gradients (5–70 °C and 5–10, respectively). Crucially, we demonstrated that products derived from such laccase and catecholoxidase activities reduced significantly the numbers of colony-forming units of both Gram-positive and Gram-negative bacteria *in vitro*. We further screened limpet tissues for signs of melanin using wax histology, and found cells replete with eumelanin-like pigments and lipofuscin in the digestive gland, connective tissues, barrier epithelia and gills. Our data

[redacted]

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CO, catecholoxidase; CTAB, cetrimonium bromide; DHPPA, 3,5-Dihydroxyphenylpropionoic acid; L-DOPA, L-3,4-Dihydroxyphenylalanine; EDTA, ethylenediaminetetraacetic acid; 4-HA, 4-hydroxyanisole; 4-HR, 4-hexylresorcinol; PO, phenoloxidase; PPD, para-Phenylenediamine; PTU, phenylthiourea; Syringaldazine, 4-Hydroxy-3,5-dimethoxybenzaldehyde azine; TY, tyrosinase

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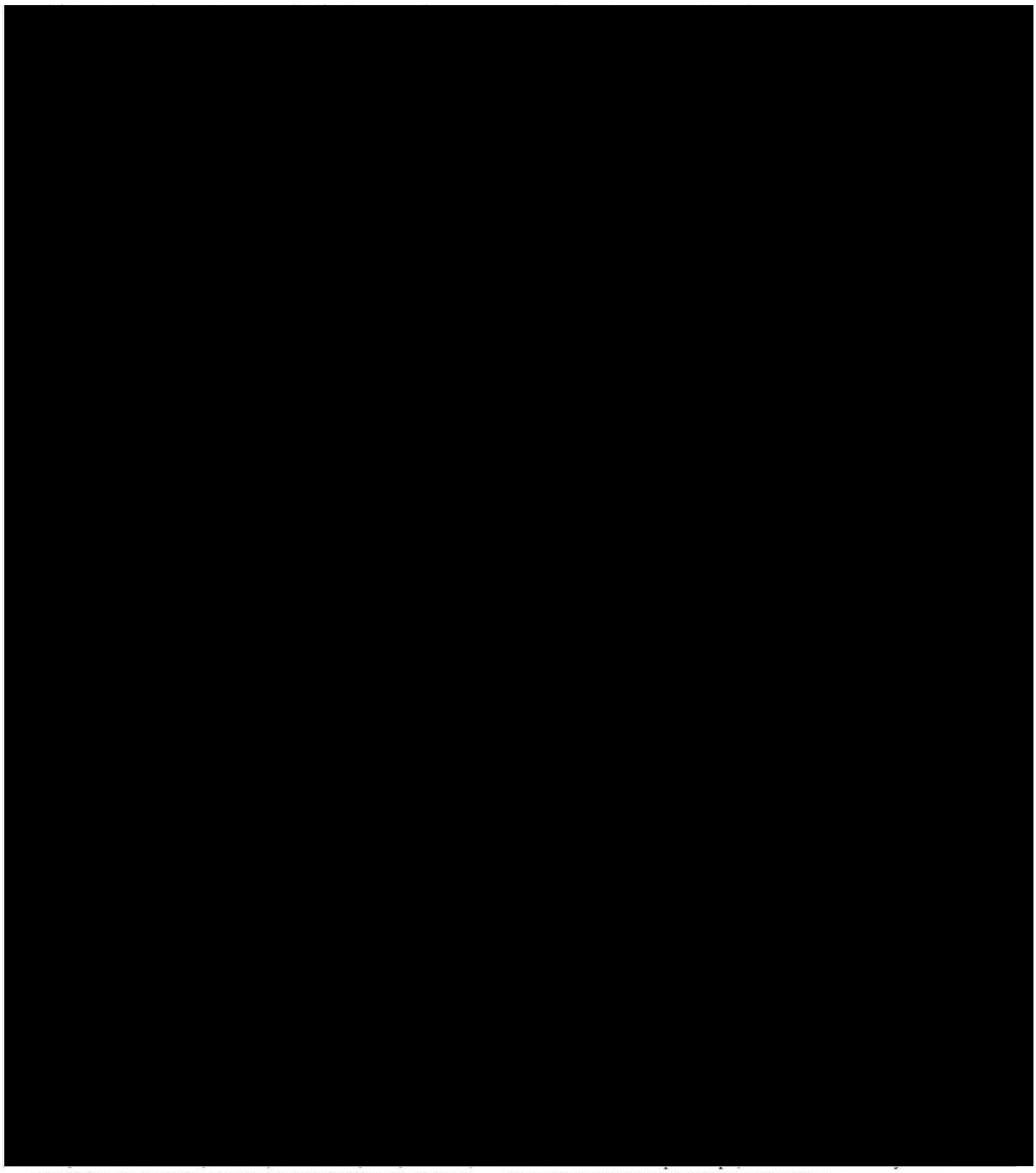
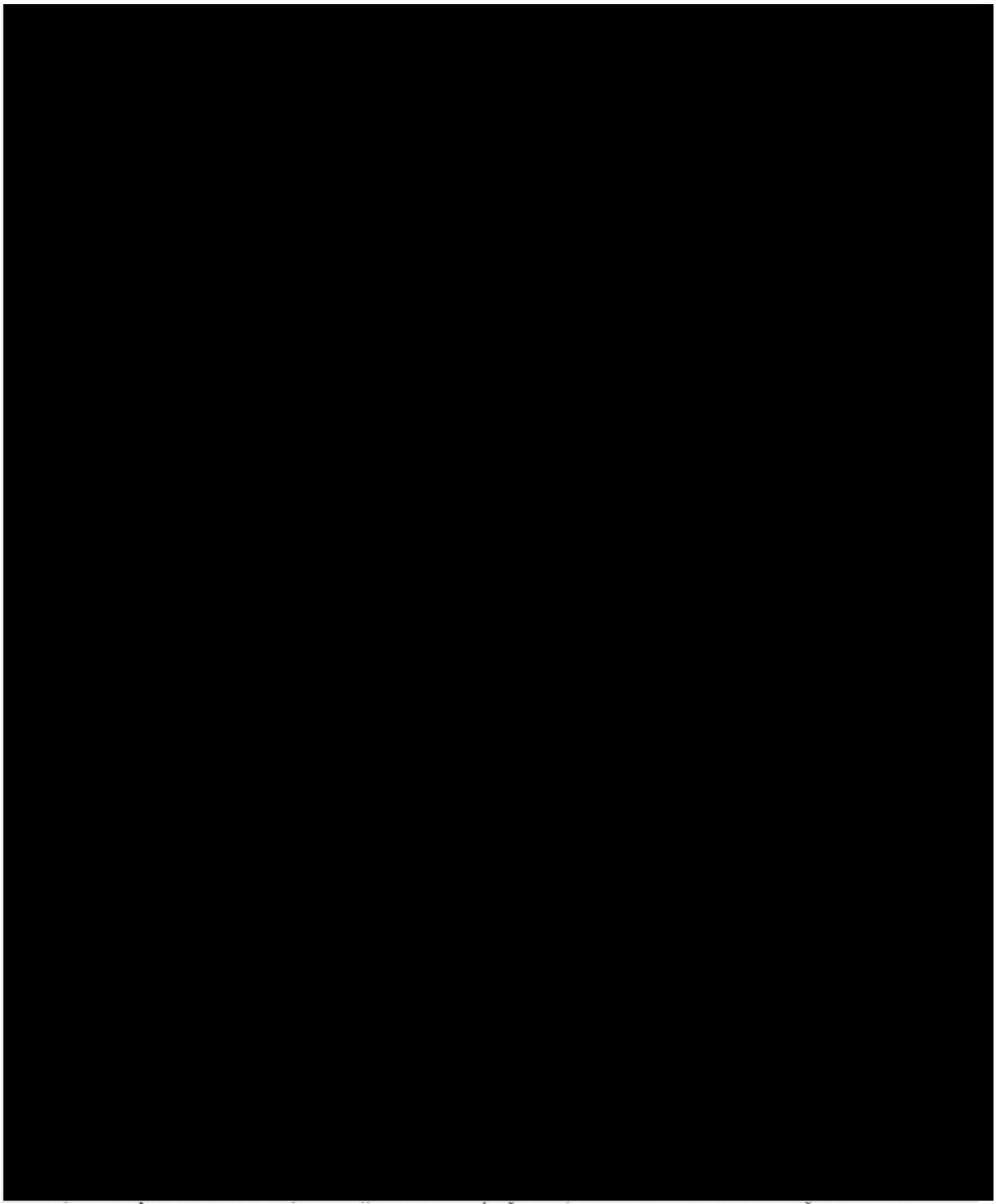


−3.987040). Samples were returned to the laboratory and processed immediately. Individuals were separated from stacks and cleaned of epibionts.

values ranging from 5 to 10 (in increments of 0.5).

To gain insight into the haemolymph pH of *C. fornicata in situ*, 141 fresh limpets were collected in March 2019. Haemolymph was isolated



attribute antibacterial activity to laccase- and catecholoxidase-derived Menten equation and Lineweaver-Burk intercepts (Table 2). Goodness

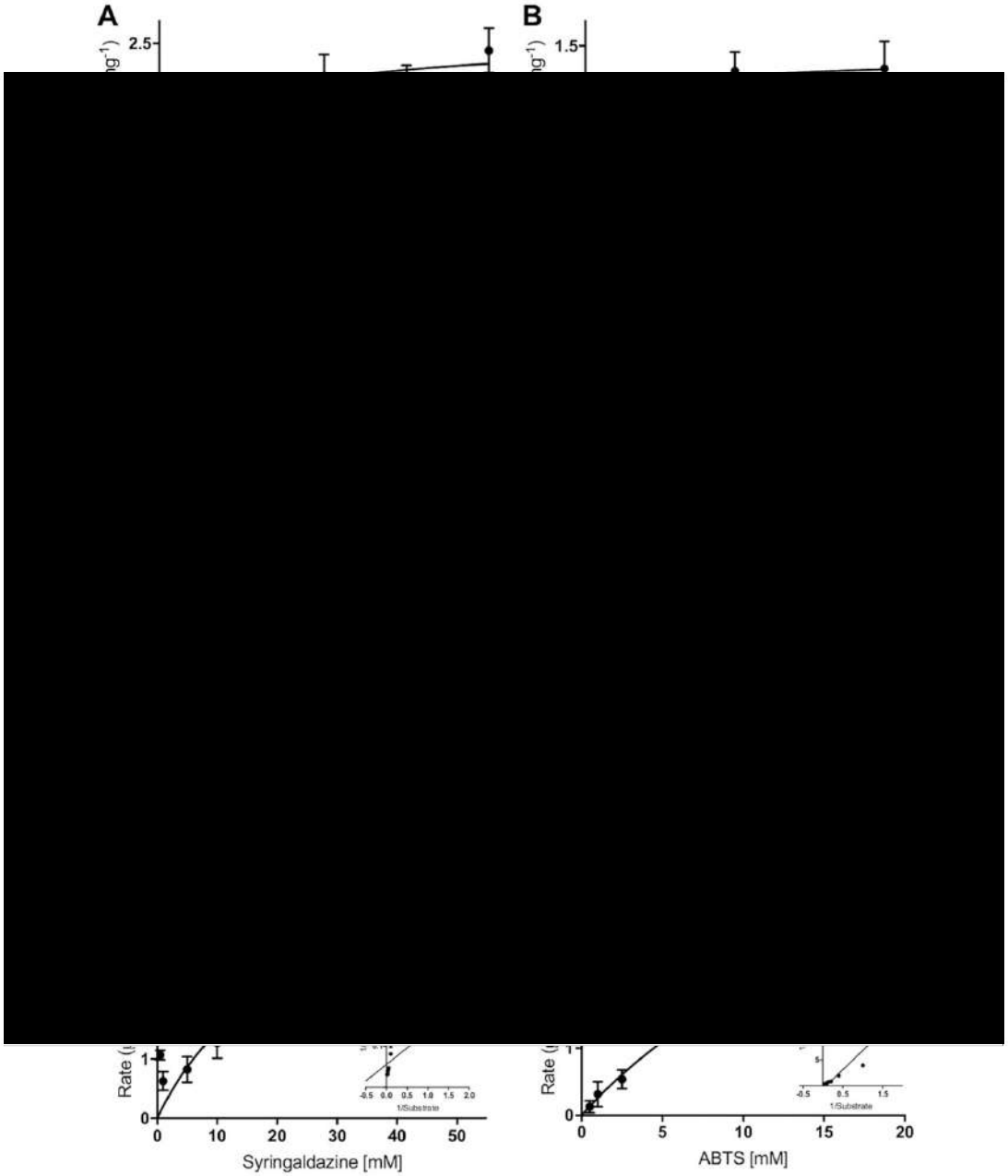


Fig. 2. Laccase and catecholoxidase activities of *Crepidula fornicata* haemolymph protein in the presence of diverse substrates *in vitro*. Protein (1 mg mL^{-1}) was incubated in the presence of each substrate for 10 min. Products derived from the enzymatic oxidation of substrate were observed across several wavelengths (listed in Table 1). Values represent the mean ± standard error ($n = 3$ biological replicates made-up of 3 technical replicates each). Enzyme-substrate kinetics were calculated in GraphPad PRISM v7 using Michaelis-Menten non-linear regression. Each panel also contains the respective double-reciprocal (Lineweaver-Burk) plot. Inset – chemical structures of *ortho*-diphenols (coloured black), *para*-diphenol (coloured blue), phenols with methoxy groups (coloured grey), and a non-phenolic substrate (coloured red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

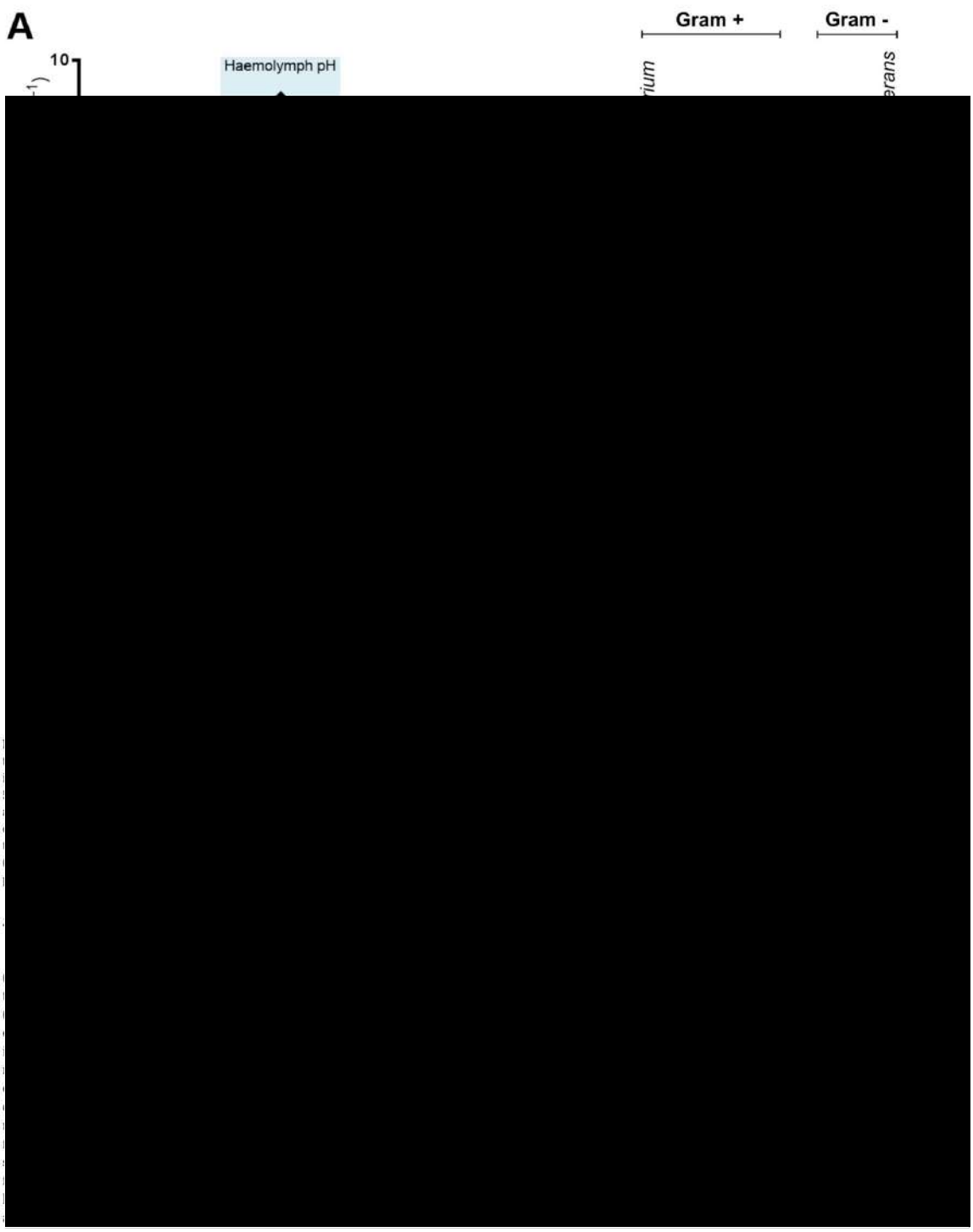
Table 2
Kinetic properties of laccase and catecholoxidase activities.

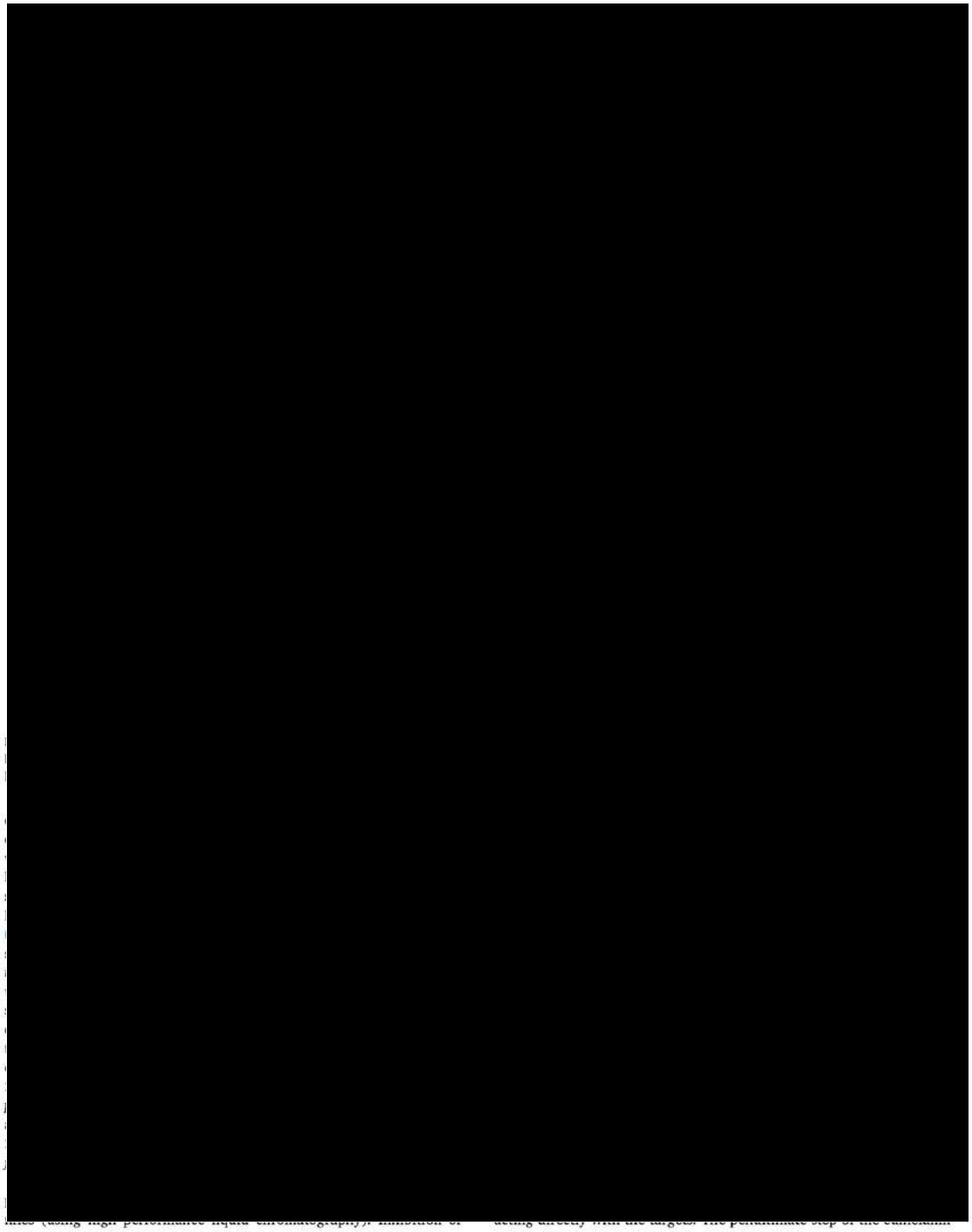
Substrate	Substrate class	Enzyme	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	R^2
ABTS	methoxy-phenol	Laccase	21.1 ± 4.82	5.71 ± 0.81	0.96

[redacted]

[a] substrates were used at a standard concentration of 5 mM for all inhibition assays.

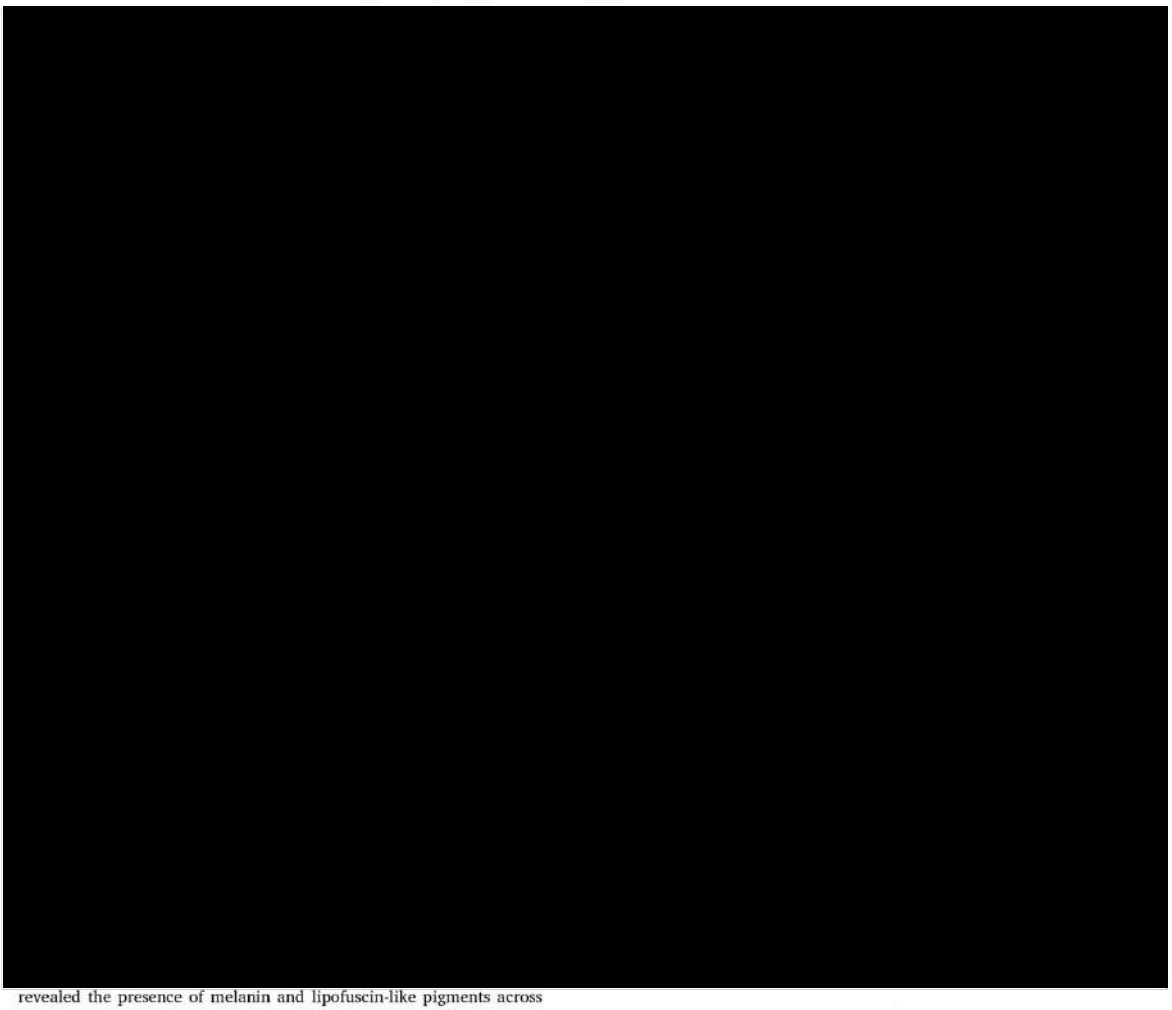
use of L-DOPA did lead to decreases in *B. megaterium* and *B subtilis* CFUs, neither were significantly different to the respective controls ($P = 0.099$ and $P = 0.335$; Supplementary Fig. 2).


A
Gram +
Gram -
10
Haemolymph pH



tyrosine hydroxylase and dopamine-β-hydroxylase using α-methyl-DL-m-tyrosine and diethyldithiocarbamate reduced levels of

synthesis pathway is 5,6-Dihydroxyindole (DHI) formation, which can happen spontaneously or enzymatically from DOPA-derivatives, and is



revealed the presence of melanin and lipofuscin-like pigments across diverse tissue types. In previous work by Tiley et al. (2018, 2019), brown inclusion bodies – bulbous or conical in shape – were characterised in the digestive gland of another gastropod, the queen conch *Lobatus gigas*. Using a combination of techniques, including histochemical staining and electron microscopy, these were confirmed to be aggregates of melanin, iron, glycoproteins and mucopolysaccharides. In line with our observations of slipper limpet tissues, Tiely et al. (2018, 2019) did not find any evidence of damage, inflammation or infection (e.g., apicomplexan parasites), however, they did observe such pigmented deposits in several other areas, including ganglia. These studies may go some way to explain the presence of lipofuscin – a lysosomal degradation product in the digestive gland and connective tissues of *C. fornicata* (Fig. 5). Lipochrome in the form of small yellow aggregates can be considered stage 1 lipofuscin, which can go on to form immature (stage 2) brown bodies. These brown bodies are often associated with pathogen clearance, mineral storage and cellular senescence, and the darker pigmentation can be attributed to melanin accumulation from oxidation reactions (Valembois et al., 1994).

The published genomes of several bivalves, *C. gigas* (Zhang et al., 2012), *C. farreri* (Li et al., 2017) and *Pinctada fucata martensii* (Du et al.,

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Zhao, P., Lu, Z., Strand, M.R., Jiang, H., 2011. Antiviral, anti-parasitic, and cytotoxic effects of 5, 6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. Insect Biochem. Mol. Biol. 41 (9), 645–652.
Zhou, Z., Ni, D., Wang, M., Wang, L., Wang, L., Shi, X., et al., 2012. The phenoloxidase activity and antibacterial function of a tyrosinase from scallop *Chlamys farreri*. Fish Shellfish Immunol. 33 (2), 375–381.

Contribution to the following work:

Parasitology

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Detection of haplosporidian protistan parasites supports an increase to their known diversity, geographic range and bivalve host specificity

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Research Article

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Abstract

Haplosporidian protist parasites are a major concern for aquatic animal health, as they have been responsible for some of the most significant marine epizootics on record. Despite their impact on food security, aquaculture and ecosystem health, characterizing haplosporidian diversity, distributions and host range remains challenging. In this study, water filtering bivalve species, cockles *Cerastoderma edule*, mussels *Mytilus* spp. and Pacific oysters *Crassostrea gigas*, were screened using molecular genetic assays using deoxyribonucleic acid (DNA) markers for the Haplosporidia small subunit ribosomal deoxyribonucleic acid region. Two Haplosporidia species, both belonging to the *Minchinia* clade, were detected in *C. edule* and in the blue mussel *Mytilus edulis* in a new geographic range for the first time. No haplosporidians were detected in the *C. gigas*, Mediterranean mussel *Mytilus galloprovincialis* or *Mytilus* hybrids. These findings indicate that host selection and partitioning are occurring amongst cohabiting bivalve species. The detection of these Haplosporidia spp. raises questions as to whether they were always present, were introduced unintentionally *via* aquaculture and or shipping or were naturally introduced *via* water currents. These findings support an increase in the known diversity of a significant parasite group and highlight that parasite species may be present in marine environments but remain undetected, even in well-studied host species.

Introduction

The phylum Haplosporidia consists of 36 recognized species in four genera, *Urosporidium*, *Minchinia*, *Haplosporidium* and *Bonamia*. Certain haplosporidian species have been credited with causing some of the most serious epizootic marine disease breakouts on record, in particular in shellfish species (Carnegie *et al.*, 2016). In recent years, over ten newly detected haplosporidian species have been added to the phylum, including species in the *Bonamia* and *Minchinia* lineages (Arzul and Carnegie, 2015). In a previous study, environmental samples (water and sediment) from South Africa, Panama and the UK were molecularly screened, and revealed previously undescribed phylogenetic lineages within the Haplosporidia (Hartikainen *et al.*, 2014). Besides their low detection prevalence, a major reason for the lack of detection of novel haplosporidian taxa is thought to be the use of (and increasing reliance on) broadly targeted molecular probes that are unsuitable for the highly divergent genes that characterize parasite groups (Hartikainen *et al.*, 2014). Despite these obstacles, novel haplosporidians continue to be discovered in host/carrier species and habitats including the recently detected *Haplosporidium pinnae* in the fan mussel *Pinna nobilis* in the western Mediterranean Sea, thus highlighting the possibility that a significant diversity of haplosporidians have yet to be discovered (Lynch *et al.*, 2013, 2014; Arzul and Carnegie, 2015; Pagenkopp Lohan *et al.*, 2016; Ramilo *et al.*, 2017; Catanese *et al.*, 2018).

Recent discoveries have highlighted that the geographic range of Phylum Haplosporidia is much greater than originally appreciated. *Bonamia ostreae*, which has caused significant mortalities in the European flat oyster *Ostrea edulis*, was thought to exclusively occur in the Northern hemisphere in both western and eastern North America and Europe but is now known to extend to the southern hemisphere in New Zealand where it has parasitized the native oyster *Ostrea chilensis* (Lane *et al.*, 2016). *Bonamia exitiosa* was originally described in *O. chilensis* in New Zealand (Dinamani *et al.*, 1987; Hine, 1996) but is now known to have an extensive geographic range in the southern and northern hemisphere and can infect several oyster species (Hill-Spanik *et al.*, 2015). *Haplosporidium nelsoni*, the causative agent of MSX disease in the eastern oyster *Crassostrea virginica* in North America, was detected for the first time in Irish and Spanish Pacific oysters *Crassostrea gigas* and in *O. edulis* in Ireland (Lynch *et al.*, 2013). In addition, *Minchinia mercenariae*, reported to cause infections in the hard clam *Mercenaria mercenaria* from the Atlantic coast of the United States (Ford *et al.*, 2009), was detected in the common cockle *Cerastoderma edule* in the Netherlands

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(Engelsma *et al.*, 2014) and the UK (Longshaw and Malham, 2013) where it was implicated in host population crashes, and an *M. mercenariae*-like parasite was recently confirmed in *C. edule* in Galicia, Spain (Ramilo *et al.*, 2017).

Parasites in the phyla Haplosporidia have been reported infecting a number of bivalve hosts from across Europe. Species known to infect *C. edule* are *Haplosporidium edule, Minchinia tapetis* and *M. mercenariae* (Longshaw and Malham, 2013; Ramilo *et al.*, 2017) and in mussels *Minchinia* sp. in *Mytilus galloprovincialis* along the Mediterranean coast of France (Comps and Tige, 1997), *Haplosporidium* sp. in *M. edulis* in Maine (Figueras and Jardon, 1991), USA, a haplosporidian-like parasite in *M. edulis* in Atlantic Canada (Stephenson and McGladdery, 2002) and *Minchinia mytili* in *Mytilus edulis* (Ward *et al.*, 2019). Lynch *et al.* (2014) assessed the health status of *Mytilus* spp. around the coasts of Ireland and Wales, and detected a previously undescribed haplosporidian (Haplosporidia sp. SAL-2014) belonging to the *Minchinia* clade in a single *M. edulis* from Wales (Lynch *et al.*, 2014). The sequence of this haplosporidian was most similar to *Minchina chitonis* detected in the chiton *Lepidochitona cinereus* and an undescribed haplosporidian species parasitizing the Florida marsh clam *Cyrenoida floridana* (Reece *et al.*, 2004). *H. nelsoni* has been associated with *C. gigas* populations in California (Friedman, 1996; Burreson *et al.*, 2000), Korea (Kern, 1976), France (Renault *et al.*, 2000), Japan (Friedman, 1996; Kamaishi and Yoshinaga, 2002) and Ireland (Lynch *et al.*, 2013). In addition to *H. nelsoni*, *Haplosporidium costale*, a species associated with seaside organism (SSO) disease in *C. virginica*, was recently detected in *C. gigas* in China for the first time (Wang *et al.*, 2010).

The objectives of this study were: determine (1) if Haplosporidia spp. were present in cockles, mussels and oysters at particular sites in Ireland, (2) did coinfection occur and (3) what abiotic and biotic factors associated with site influence and anthropogenic activities, i.e. aquaculture and shipping may influence their presence.

Materials and methods

Study sites, bivalve spp. sampled and site description

A range of Irish coastal sites was sampled with different environmental (abiotic and biotic) factors and anthropogenic influences (aquaculture and shipping) from nature reserve sites to key economic areas influenced by frequent and heavy anthropogenic effects (Table 1, Fig. 1). Multiple samples of cockles ($n = 1{,}604$), mussels ($n = 516$) and oysters ($n = 420$) were collected from five of the fourteen Irish sites over several months and years resulting in a higher overall number of individuals being screened at those sites. A minimum sample size of thirty individuals was collected on a single occasion from five other sites.

Cockle, mussel and pacific oyster samples

Wild *C. edule* was sampled at a nonculture site (March 2010–June 2011) and at two *C. gigas* culture sites (April to August 2015) (Table 1). Cultured *C. gigas* were sampled from both culture sites in 2015. Wild *Mytilus* spp. were sampled from both culture sites in 2015, from a third culture site in September 2017 and from 11 nonculture sites in May to November 2017.

Histology

A cross section of tissue (mantle, gill, connective, digestive and gonad) was taken from each cockle, mussel and oyster, and was fixed in Davidson's solution for 24–48 h and subsequently stored in 70% ethanol. The fixed tissue was then dehydrated fixed in paraffin cut at 5 μm and stained using haematoxylin and eosin (H&E) and mounted in dibutyl phthalate xylene. Slides were examined using a Nikon light microscope at 4×, 10×, 20×, 40× and 100× magnification.

Molecular genetic diagnostic techniques

Genomic deoxyribonucleic acid (DNA) from *C. edule*, *Mytilus* spp. and *C. gigas* gill tissue (~5 mm^2 from fresh and fresh frozen (−20 °C)) was extracted from each individual using the Chelex-100 extraction method (Walsh *et al.*, 1991).

Several polymerase chain reactions (PCRs) using generic and specific primers and thermocycling conditions for Haplosporidia spp. were utilized in the molecular genetic screening. Additionally, a PCR to detect the nuclear DNA markers Me15/Me16 (Inoue *et al.*, 1995) was carried out on mussels that amplified a PCR product to determine if they were *M. edulis*, *M. galloprovincialis* or hybrids of both parent species.

Two generic PCRs using similar mastermix and thermocycling conditions with primer pair HAP-F1′/HAP-R3′ (Renault *et al.*, 2000; Lynch *et al.*, 2014) and ssu980/HAP-R1′ (Molloy *et al.*, 2012; Lynch *et al.*, 2014) to amplify the Small subunit ribosomal deoxyribonucleic acid (SSU rDNA) region of Haplosporidia spp. were carried out on *C. edule*, *Mytilus* spp. and *C. gigas* genomic DNA. A third PCR using specific *H. nelsoni* MSX-A′/MSX-B′ primers to amplify the small subunit ribosomal ribonucleic acid (SSU rRNA gene) and similar mastermix and thermocycling conditions (Renault *et al.*, 2000) was carried out on DNA from *C. gigas*. Negative controls containing double distilled water (ddH_2O) were used in each PCR to control for contamination and infected Haplosporidia (*B. ostreae*, *H. nelsoni*, Haplosporidia sp. SAL-2014) genomic DNA was used as a positive control. Initially, in the screening of cockles from 2010/2011 no *M. mercenariae*-like positive material was available as it had not been detected before, however amplification in that single PCR occurred with the cockle deemed positive in the histology and that cockle's DNA was subsequently used as the positive control in the screening of the 2015 samples.

Electrophoresis of amplified products was carried out in a 2% agarose gel and was run with an electrical current of 110 V for 45 min. The expected product size for the HAP-F1′/HAP-R3′ PCR was 350 bp (Renault *et al.*, 2000), for the ssu980/HAP-R1′ was 430 bp (Molloy *et al.*, 2012) and for the MSX-A′/MSX-B′ PCR was 573 bp (Renault *et al.*, 2000).

Pooled PCR products using replicates (×3) from individual cockles [Flaxfort ($n = 1$), Dungarvan ($n = 1$) and Carlingford ($n = 1$)] using the HAP-F1′/HAP-R3′ primers and mussels (Clonea, $n = 1$) using the ssu980/HAP-R1′ primers were used to increase the amplicon concentration for Sanger sequencing, as recommended by EurofinsMWG. Both forward and reverse DNA sequences that were optimally generated by EurofinsMWG Sanger sequencing laboratory were matched against the National Center for Biotechnology Information (NCBI) nucleotide database with Basic Local Alignment Search Tool (BLASTn), which finds regions of local similarity between sequences to identify and confirm the DNA being detected in the PCRs. Percentage query coverage in BLAST refers to how much of the query sequence is aligned with results from the database sequence or, in other terms, the size of the sequence fragments that are comparable, while % identity measures the extent to which the nucleotide sequences relate to one another.

Phylogenetic analysis

18S SSU rDNA sequence data for 28 operational taxonomic units from GENBANK (Table 2) were downloaded, to which, the two 18S sequences were added. These data were aligned using Clustal Omega (Sievers *et al.*, 2011) at the European Bioinformatics Institute portal (https://www.ebi.ac.uk/Tools/msa/clustalo/). The final alignment was 2221 bp in length. The best-fit

Table 1. Description of bivalve species, sample sites, months, years and anthropogenic activities at each site, and histology and molecular analyses for Haplosporidia spp

Site # (Fig. 1)	Site	Anthropogenic activity	Month	Year	*n*	PCR+	Histology+	Sequencing
			Wild C. edule					
13	Flaxfort	Rural/agriculture	March–June	2010 to 2011	900	1.7% (1/60)	0.11% (1/900)	1/Isolate221 (A#KY522823.1)
5	Dungarvan[a]	Aquaculture & agriculture	April–August	2015	250	4% (10/250)	50% (10/20)	1/Isolate226 (A#KY522823.1)
1	Carlingford[a]	Aquaculture & shipping	April–August	2015	454	10.8% (49/454)	100% (20/20)	1/Isolate228 (A#KY522823.1)
	Total				*1604*	7.9% (60/764)	3.3% (31/940)	
			Wild Mytilus spp.					
1	Carlingford[a]	Aquaculture & shipping	April–August	2015	120	0/120	0/120	
2	Annestown	Rural/agriculture	September	2017	30	0/30	0/30	
3	Stradbally	Rural/agriculture	June	2017	30	0/30	0/30	
4	Clonea	Rural/agriculture	September	2017	60	1.7% (1/60)	1.7% (1/60)	1/Isolate376 (A#KC882876)
5	Dungarvan[a]	Aquaculture & agriculture	September	2015 & 2017	276	0/276	0/276	
6	Helvic Head[a]	Aquaculture & agriculture	June	2017	30	0/30	0/30	
7	Ballysquinn[a]	Rural/agriculture	June	2017	30	0/30	0/30	
8	Whiting Bay	Rural/agriculture	July	2017	30	0/30	0/30	
9	Ballymacoda[a]	Aquaculture & agriculture	June & November	2017	60	0/60	0/30	
10	Roches point	Shipping		2017	60	0/60	0/60	
11	Ringaskiddy	Urban & shipping	July & October	2017	60	3.3% (2/60)	3.3% (2/60)	
12	Garrettstown	Rural/agriculture	May	2017	70	0/70	0/70	
13	Flaxfort	Rural/agriculture	June	2017	60	0/60	0/60	
14	Lough Hyne[a]	MNR/rural/agriculture	May	2017	60	0/60	0/60	
	Total				*976*	2.5% (3/120)	2.5% (3/120)	
			Cultured C. gigas					
5	Dungarvan[a]	Aquaculture & agriculture	April–August	2015	180	0/180	0/60	
1	Carlingford[a]	Aquaculture & shipping	April–August	2015	240	0/240	0/60	
	Total				*420*			

+ Positive detection.
A# denotes Genbank Accession Number exact match to.
[a]Special Areas of Conservation (SAC) and Special Protected Areas (SPA) under the birds and habitats EU Directive.

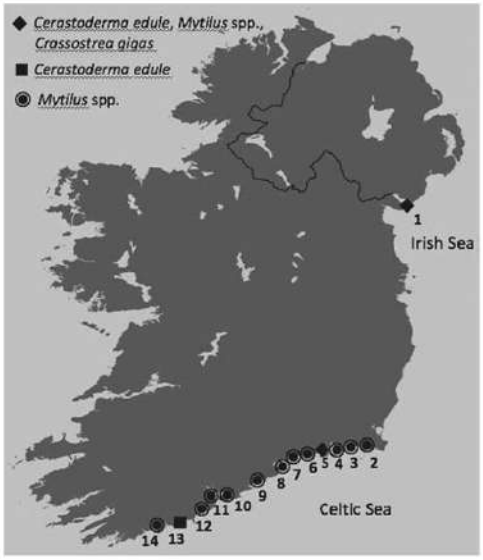

Fig. 1. Map of Ireland showing the sample sites and bivalve species sampled and screened for Haplosporidia spp. at each location during this study.

evolutionary model for the aligned sequence data was assessed in the jModelTest (v2.1.10; Darriba *et al.*, 2012), using the small sample corrected Akaike information criterion (Hurvich and Tsai, 1989). This returned the generalized time reversible (GTR) model, with a four-category gamma rate distribution and invariant sites (GTR + G + I) as the best-fit model.

The phylogeny was reconstructed in Mr Bayes (v3.2.5; Ronquist *et al.*, 2012). A total of 379 base pairs in three distinct regions of the alignment were not able to be unambiguously aligned, and were excluded from the analysis. Two runs of four chains each were run for 5 000 000 generations, saving every thousandth tree. Nodal posterior probabilities were assessed using the 50% consensus tree topology (Huelsenbeck *et al.*, 2002), discarding the first 25% of trees as burnin. Aligned sequences and commands used in the phylogenetic analysis are provided in ***.nex (Supplementary Information).

Resulting forward and reverse sequences were aligned and manually edited to resolve any ambiguities in base calling. The resulting alignments were matched against the NCBI nucleotide database with BLASTn.

Statistics

A χ^2 test was used to determine whether prevalence differences of parasites observed were significant (P values < 0.05) between sample sites in 2015. R packages used were dplyr, tidyr, ggplot2, car and NCStats.

Results

Histology

Cockles

Haplosporidia-like single cell and plasmodia-like life stages identical to those described in Ramilo *et al.* (2017) and a spore-like stage (Fig. 2A–F) were observed in the connective tissues of Irish *C. edule* in a single individual at Flaxfort the nonculture site in June 2010 [0.11% (1/900)]. Subsequently in 2015, a subsample of PCR positive *C. edule* at Dungarvan (100% prevalence in the subsample of 10 PCR positive individuals) had positive detection of Haplosporidia-like cells in the corresponding histology section. Similarly in 2015 at Carlingford, Haplosporidia-like cells were observed in the tissues (mainly in the gill, gonad and digestive area, with some physical disruption to the tissues and infiltration of haemocytes) of a subsample of 20 PCR positive individuals (Table 1).

Table 2. Operational taxonomic units from GENBANK used in the phylogenetic analysis

Genbank accession number	Taxon
AF174374	*Massisteria marina*
AF101052	*Cercomonas longicauda*
X77692	*Euglypha rotunda* strain CCAP 1520/1
HGU42447	*Heteromita globosa*
CAU42449	*Cercomonas* sp.
CAU42450	*Cercomonas* sp.
AY449715	Haplosporidian parasite of *Pandalus platyceros*
AY449716	Haplosporidian parasite of *Pandalus platyceros*
AF492442	Haplosporidian parasite of *Haliotis iris*
AY449714	Urosporidium parasite of *Stictodora lari*
UCU47852	*Urosporidium crescens*
HLU47851	*Haplosporidium louisiana*
HNU19538	*Haplosporidium nelsoni*
AF387122	*Haplosporidium costale*
AY452724	*Haplosporidium pickfordi*
AY449713	*Haplosporidium lusitanicum*
AF262995	*Bonamia ostreae*
AF192759	*Bonamia ostreae*
AF337563	*Bonamia* sp.
AF508801	*Bonamia roughleyi*
AY449710	*Minchinia tapetis*
FJ518816	*Minchinia_mercenaria*
KY522821	*Minchinia* sp. Clone 1
KY522823	*Minchinia* sp. Clone 3
AY449711	*Minchinia chitonis*
MTU20319	*Minchinia teredinis*
AY449712	Haplosporidian parasite of *C. floridana*
EF165631	*Minchinia occulta*
	Isolates 221, 226 & 228 – this study, isolate 376 – this study

Mussels

In the *M. edulis*, haplosporidia-like single cell and plasmodia life stages similar to those described in the cockles were also observed in mussels deemed to be positive in the PCR (Clonea 1.7% (1/60) and Ringaskiddy 3.3% (2/60)). No spores were observed (Table 1).

Molecular genetic screening:

Cockles: A single cockle at Flaxfort Strand deemed to have haplosporidian-like cells visualized in the histology (0.11% prevalence, 1/900) also produced a PCR product (350 bp) (1.7%

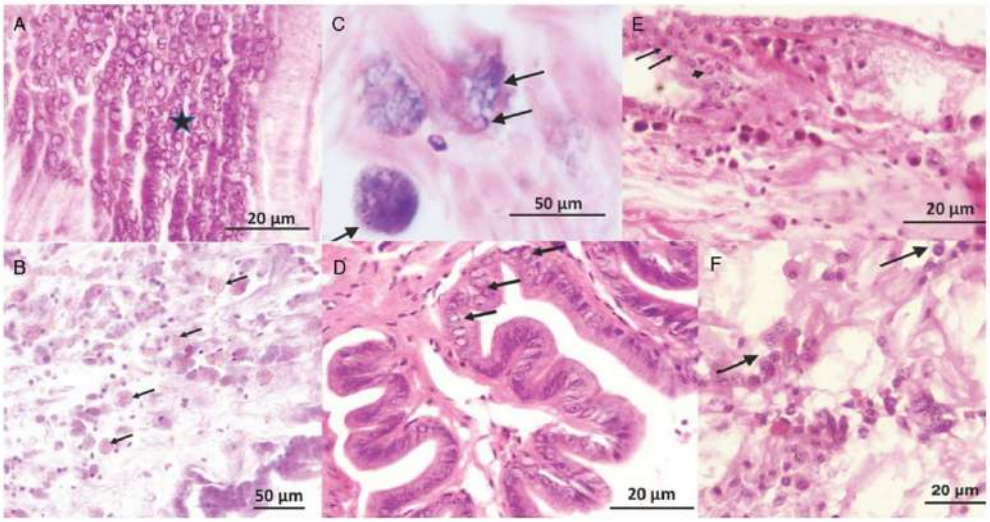

Fig. 2. (A) Large number of haplosporidian spores in the connective tissues of *C. edule*, (B) & (C) multiple haplosporidia-like sporonts and developing spores respectively in the connective tissues of *C. edule*, (D) spores in the mantle tissue of *C. edule*, (E) uninucleate 'fried egg' (arrows) and binucleate (arrow head) cells in the epithelium of *C. edule* and (F) multiple plasmodia in the connective tissues of *C. edule*.

prevalence, 1/60) with the HAP-F1′/HAP-R3′ primers (Renault *et al.*, 2000) in June 2010.

Overall, 59 (8.3%, (59/704)) cockles produced a PCR product (350 bp) with the HAP-F1′/HAP-R3′ primers (Renault *et al.*, 2000) from April to August 2015 at both oyster culture sites – the total prevalence was 4% (10/250) in Dungarvan and 10.8% (49/454) in the Carlingford cockles, which was statistically different ($\chi^2 = 14.381$, df = 1, P value < 0.001). The highest prevalence was detected in the Carlingford cockles in July and in the Dungarvan cockles in April (Fig. 3).

A single haplosporidian spp. *M. mercenariae-like* parasite in *C. edule* at Flaxfort Strand (2010) and at the *C. gigas* culture sites Dungarvan and Carlingford Lough (2015) was identified in the PCR products amplified in cockles from the three sites [Accession # KY522823.1 (Ramilo *et al.*, 2017)] with 97% query coverage and 100% maximum identity and are referred to as 'Isolates 221, 226 and 228' in this study.

Mussels: No PCR products were produced using both primer pairs (Renault *et al.*, 2000; Molloy *et al.*, 2012) in the wild *Mytilus* spp. screened at the three culture sites at Dungarvan, Carlingford and Ballymacoda.

Overall, of the 11 nonculture sites screened two sites with 0.5%, (3/580) of mussels produced products (430 bp) using the ssu980/HAP-R1′ (Molloy *et al.*, 2012). A total of 6.6% (2/60) of *M. edulis* from two Ringaskiddy samples [3.3% each, (1/30)] produced products (430 bp) in July 2017 and October 2017 respectively, while a single *M. edulis* (3.3%, 1/30) at Clonea in September 2017 produced a PCR product. All of these products were identified as Haplosporidia sp. SAL-2014 [Accession # KC852876.2 (Lynch *et al.*, 2014)] with 98% query coverage and 100% maximum identity and are referred to as 'Isolate 376' in this study.

Pacific oysters:

No PCR products were produced using either primer pairs (Renault *et al.*, 2000; Molloy *et al.*, 2012) in the *C. gigas* screening or in the MSX-A′/MSX-B′ screening for *H. nelsoni* (Renault *et al.*, 2000).

Direct sequencing:

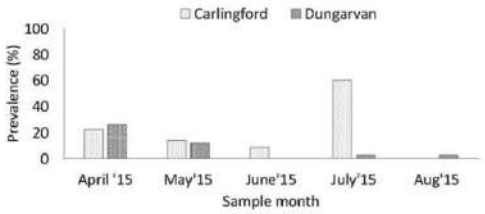

Fig. 3. Prevalence of *M. mercenariae*-like parasite in *C. edule* at the two Irish *C. gigas* culture sites *from April to August 2015*.

The PCR product of the single mussel at Clonea was successfully sequenced however, the PCR products for both mussels at Ringaskiddy were also sent for sequencing but only the reverse sequences (which were a match) were amplified and not the corresponding forward sequence. As both sequences could not be aligned the result was not considered robust.

Due to the cost of sequencing all of the cockle PCR products amplified ($n = 60$), a subsample of cockle PCR products was sent for sequencing (representative of each sample site and years). Additionally, a similar morphology of each parasite was observed in the cockle and mussel histology for each respective parasite. More recent sequencing of PCR products ($n = 40$) from additional cockles at each location (study currently being carried out by the authors) has confirmed the findings of this study.

Phylogenetic analyses:

The maximum likelihood phylogeny of haplosporidian taxa conclusively places Isolate 376, which is identical to the undescribed isolate SAL-2014 [Haplosporidia sp. Accession # KC852876.2 (Lynch *et al.*, 2014)], and Isolates 221, 226 and 228, which are identical to *M. mercenariae-like* parasite [Accession # KY522823.1 (Ramilo *et al.*, 2017)], in a clade with species of the genus *Minchinia* (Fig. 4). Also included in this clade is an undescribed haplosporidian parasite of the Florida marsh clam *C. floridana*. The internal topology of this clade is

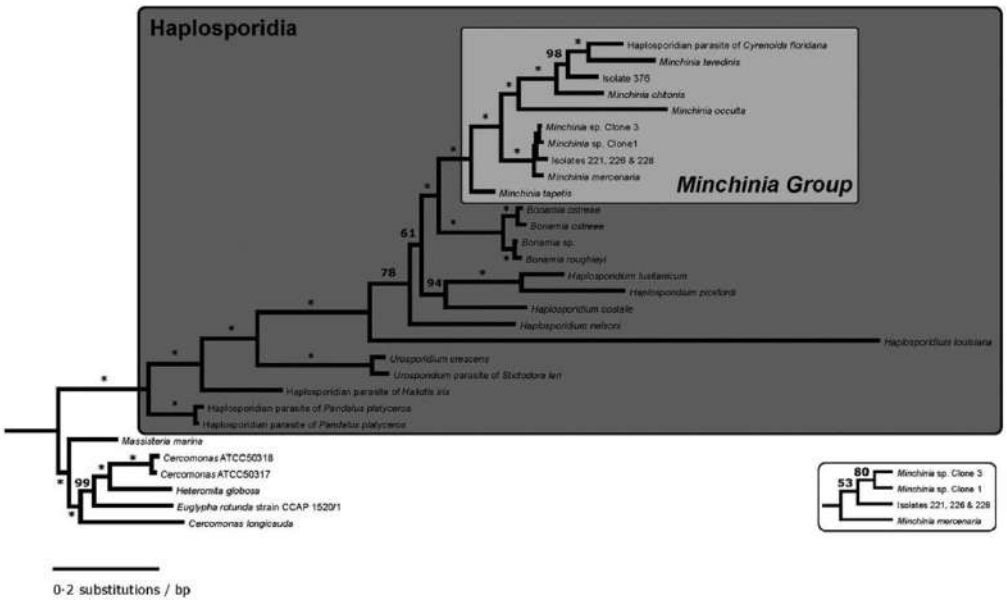

Fig. 4. Bayesian phylogenetic tree of haplosporidian taxa based on partial 18S SSU rRNA sequences. Branches marked with an asterisk (*) have 100% posterior probability support for that node, otherwise, the nodal support is indicated by the number given. Inset to the bottom right repeats, for clarity, the *Minchinia* subclade with very small branch lengths, and gives the nodal posterior probabilities for this topology. A well-supported clade includes all of the 18S SSU rDNA sequences assigned to the genus *Minchinia*, the previously identified parasite of *C. floridana* (Reece *et al.*, 2004), as well as the novel sequences for this study. As has been found in other analyses (Reece *et al.*, 2004; Lynch *et al.*, 2013; Ramilo *et al.*, 2017), we find strong evidence for the paraphyly of the genus *Haplosporidium*.

fairly well-resolved, with Isolate 376 and the *C. floridana* parasite being recovered with *M. chitonis* and *M. teredinis* with unanimous bootstrap support. The phylogeny also recovers unambiguous support for the monophyly of *Urosporidium* (Fig. 4). Several of the internal nodes of the phylogeny are poorly supported in the bootstrap (Fig. 4), although, these involve branching events among the *Bonamia* Group, the *Minchinia* Group and the paraphyletic genus *Haplosporidium*.

Discussion

Detection of Haplosporidia spp.

Two haplosporidian species were detected for the first time in *C. edule* and in *M. edulis* in a new geographic range. When detected, both Haplosporidia spp. were observed in individual samples of mussels and cockles that consisted of at least 30 individuals. The prevalence of the *M. mercenariae*-like parasite was significantly greater in *C. edule* at both aquaculture sites (late spring, summer and early autumn samples) compared to *C. edule* at the nonculture site (summer sample), which may indicate that the extended presence of this parasite has some association with anthropogenic inputs and activities in these areas. Oyster seed/spat consignments are routinely imported to both culture sites from France and the UK for on-growing in late spring. A low overall prevalence of the *M. mercenariae*-like parasite was detected in this study, which is similar to that observed in the Ramilo *et al.* (2017) study and for *M. mercenariae* infecting North American hard clams (Ford *et al.*, 2009). Haplosporidia sp. SAL-2014 (Lynch *et al.*, 2014), a novel species first detected in a Welsh *M. edulis* in 2012, was also detected in this study for the first time at a similar prevalence in wild Irish *M. edulis* at two nonculture sites. One of those sites is a busy ferry/shipping terminal in Cork Harbour and a ferry between Wales and Cork Harbour was in operation from the 1980s up until recently.

Host partitioning

Interestingly the *M. mercenariae*-like parasite was not detected in the cohabiting *C. gigas* nor was it detected in the cohabiting *Mytilus* spp. Haplosporidia sp. SAL-2014 was exclusively detected in *M. edulis* even though *M. galloprovincialis* and *Mytilus* hybrids were present at the Irish sites where it was detected. This difference in emerging haplosporidian species detection, diversity and abundance in these three bivalve species strongly indicates that these parasite species are host specific and host partitioning is occurring. Additionally, the findings of this study would indicate that the haplosporidian species may be associated more with one environmental niche than another i.e. the sediment rather than with the water column for the *M. mercenariae*-like parasite, as the cockles were collected on the surface of the sediment and would normally be buried within the sediment similar to clams, while the oysters and mussels were at least 30 cm above the sediment on trestles or rocky outcrops respectively. Hartikainen *et al.* (2014) identified three new *Minchinia*-affiliated SSU-types in environmental samples at a single location at Weymouth, SW England, in 2011 and 2012. Two of the *Minchina* spp. were closely related to *M. mercenariae* while the third was identical to *M. tapetis* [both parasites were associated with Welsh cockle mortalities; Longshaw and Malham (2013)]. Hartikainen *et al.* (2014) observed that *Minchinia*-affiliated SSU-types were detected in water column samples, strongly indicating a planktonic life-cycle stage (predominantly in the 0.45–20-μm size fraction) and were mostly in the April samples. Findings from this study and

the Hartikainen *et al.* (2014) study would indicate that *Minchinia* spp. are niche selective or their detections are closely associated with their life stages i.e. in the water column in a planktonic intermediate host or near the sediment associate with primary bivalve host species.

Phylogenetics

The phylogenetic analysis in this study recovers unambiguous support for the grouping of these two haplosporidian isolates into a single clade with members of the genus *Minchinia*. As such, the four isolates would support the detection of a new geographic range for both of these species within the genus *Minchinia*. This would represent a substantial increase to the known diversity of this genus, as only six species associated with host species are currently described. The low bootstrap support for the internal nodes in phylogeny involves the genus *Haplosporidium*, and the interrelationships between its species and the two main haplosporidian clades, the *Bonamia* Group and the *Minchinia* Group. *Haplosporidium* is paraphyletic (Reece *et al.*, 2004), likely representing a plesiomorphic grade from which the remaining two clades derived. The short internal branch lengths and the low resolving power in the bootstrap potentially indicate a rapid diversification among haplosporidian taxa.

Site effect and shore height influence

A higher prevalence of the *M. mercenariae*-like parasite was observed in *C. edule* at Carlingford compared to Dungarvan. Carlingford is a more sheltered site with a lot of shipping activity, while Dungarvan is an oceanic bay that experiences tidal flushing, greater water exchange and some shipping activity (Lynch *et al.*, 2014; Bookelaar *et al.*, 2018). Higher pathogen retention, prevalence and diversity occur at sheltered marine environments, as pathogens are less likely to be swept away on the tides (Lenihan, 1999; Lynch *et al.*, 2016; Bookelaar *et al.*, 2018). A higher prevalence of the *M. mercenariae*-like parasite was observed in *C. edule* at the high shore at Carlingford compared to cockles lower down the shore at the oyster trestles. *C. edule* higher up the shore may experience more stressful abiotic conditions such as air exposure, fluctuating temperatures, precipitation etc., which may make them more susceptible to infections (Wegeberg and Jensen, 2003) or it may be possible that *C. edule* at the high shore are more likely to be in contact with other host species (Lynch *et al.*, 2014).

Potential drivers of emerging parasites

It is not uncommon for parasites to be widespread in marine environments, especially along near shore coastlines (Raftos *et al.*, 2014). Coastal marine environments are very vulnerable to climate change (Holt *et al.*, 2010) and a changing marine environment can have a direct impact on the distribution, life cycle and physiological status of hosts, pathogens and vectors (Gallana *et al.*, 2013). While a change in host, pathogen or vector does not necessarily translate into a change of the disease, it is the impact of climate change on the interactions between the disease components that impact disease risk (Gallana *et al.*, 2013). In addition, natural coastal disturbance arising from storm surges and high energy systems, which are predicted to increase under future climate change conditions (IPCC, 2018), may also play their part in pathogen emergence. Storms are important episodic events that can resuspend and transport sediments and are known to cause large-scale advection (i.e. transfer of heat or matter), sediment resuspension and transport (Cacchione *et al.*, 1987; Warner *et al.*, 2008). An association between disease outbreaks in marine organisms and storm activity is recognized (Burge *et al.*, 2014). In one study, a strong correlation with hurricane activity [and a strong storm (nor'easter)] and the amoebic pathogen *Paramoeba invadens*, causative agent of urchin disease in the green urchin *Strongylocentrotus droebachiensis* in the northwest Atlantic, was modelled and confirmed (Feehan *et al.*, 2015). Other factors such as the movement of non-native species, both intentionally (i.e. for aquaculture) and unintentionally (i.e. as stowaways in ship ballast water or on hulls), brings the threat of 'pathogen spillover' into introduced areas and highly-susceptible host populations (Carnegie *et al.*, 2016; Ek-Huchim *et al.*, 2017). It is also recognized that coastal development may unbalance marine parasite-host systems (Coen and Bishop, 2015), as past emergent and resurgent diseases in wildlife appear to be associated with anthropogenic activities (Harvell *et al.*, 1999; Daszak *et al.*, 2000). Cultured bivalve breeding programmes are designed to mitigate the impacts of pathogens and may be unintentionally resulting in or expediating 'host jumping' from now less susceptible bivalve hosts to new cohabiting and highly susceptible naïve host species (Bookelaar *et al.*, 2018). Such programmes may inadvertently be making selectively bred bivalve hosts more susceptible to novel pathogens. Additionally, due to the very poor biogeographical records available for protistan parasites it is possible that these parasites evolved in these hosts at those locations and were not introduced.

Conclusions

The detection of both these Haplosporidia spp. in the *Minchinia* clade will contribute to an improved understanding of Haplosporidia diversity, prevalence, host, geographic distribution and a certain degree ecology. This study further supports that a *M. mercenariae*-like haplosporidan infects *C. edule* in Europe (Ramilo *et al.*, 2017) and that it appears to be exclusive to *C. edule* while Haplosporidia sp. SAL-2014 (Lynch *et al.*, 2014) appears to be exclusive to *M. edule*. As many other species, including protected bird species, rely on *C. edule* and *M. edulis* as a food source and the pivotal role both bivalve species play in marine coastal ecosystems, it would be beneficial to better understand the impact that these Haplosporidia spp. may or may not have on cockle and mussel populations currently and under future climate change conditions. Understanding some of the current drivers of parasite introduction, emergence and spread may facilitate a better prediction of future impacts and host or geographical range expansion of Haplosporidia under changing environmental scenarios. In particular with a parasite group such as the Haplosporidia, which have had such a devastating historical impact both commercially and ecologically.

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Conflict of interest. The authors declare that there is no conflict of interest and certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial in the subject matter or materials discussed in this manuscript.

Ethical standards. Not applicable.

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Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name Emma Quinn .....**Signature**.....[redacted].....**date 03/18**.....

Supervisor* Christopher Coates .....**Signature** [redacted].....**date** 03/18.....

Activity title Preparation for lab work on Slipper limpets, *Crepidula fornicata* .....**Base location (room no.)** 123

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No. .....

Start date of activity (cannot predate signature dates): 9/03/2018.....

End date of activity (or 'on going') 30/09/2020 .....

Level of worker (delete as applicable) PhD student.....

~~UG,~~PG~~, research assistant, technician, administration, academic staff, other (state)~~

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes/~~not applicable
Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes/~~not applicable
Approval obtained for use of radioisotopes by COS ? ~~Yes/~~not applicable

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
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See notes in handbook for help in filling in form **(Continue on another sheet if necessary)**

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 1	**Title: Preparation for lab work on Slipper limpets,** *Crepidula fornicata*
Associated Protocols #	**Description:** Autoclaving, labelling and filling tubes ready for sample collection.

Location:

circle which Bioscience and Geography Local Rules apply –

~~**Boat Field Genetic-Manipulation**~~ **Laboratory** ~~**Office/Facility Radioisotope**~~

Identify here risks and control measures for work in this environment, additional to Local Rules
Wear PPE (lab coat, gloves and goggles). Safe handling of chemicals contained in closed containers and stored appropriately according to their level of hazards, dispose of chemicals in separate container.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Ethanol	<100ml	Flammable, Irritant	C	2
3% Saline	<100ml	None	D	2
Saline + Formaldehyde mix	<5ml	Flammable, toxic, hazardous to health , corrosive	A	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low ~~**Medium**~~ ~~**High**~~

Primary containment (of product) : Chemicals are contained in sealed plastic or glass bottles.

Storage conditions and maximum duration :- Kept at room temperature in ventilated cabinets.

Secondary containment (of protocol) open bench/fume hood/special (state) :- Fume hood

Disposal e.g. autoclaving of biohazard, SU chemical disposal: SU chemical disposal

Identify other control measures – Lab coat,gloves, safety goggles, protective shores, spillage tray

Justification and controls for any work outside normal hours n/a

Emergency procedures (e.g. spillage clearance; communication methods): **First Aid Kit available in the lab. Emergency number: 333**

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker.....*Emma Quinn* .[redacted].....

Name & counter-signature of supervisor.....[redacted].....
Date..... 03/18.....

Date of first reassessment: 01/10/18 Frequency of reassessments: Annual

Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name: Emma Quinn .....**Signature**.....[redacted].....**date 03/18**.....

Supervisor*: Dr Christopher Coates .....**Signature**[redacted].....**date**03/18.....

Activity title: Slipper limpet sampling including haemolymph, muscle and fixed sample.
Base location (room no.): 123

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No. .....
Start date of activity (cannot predate signature dates): 09/03/2018.....
End date of activity (or 'on going'): 30/09/2020 .....

Level of worker (delete as applicable): PhD student.....

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes~~/not applicable
Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes~~/not applicable
Approval obtained for use of radioisotopes by COS ? ~~Yes~~/not applicable

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
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See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 2	**Title: Slipper limpet (*Crepidula fornicata*) sampling protocol**
Associated Protocols #.....	**Description: Collect samples of hemolymph and tissue from Slipper limpet *Crepidula fornicata*, which will be stored and fixed for hemocytes counts, histology, microbial plating, immunoassays, and molecular analysis**

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat Field Genetic Manipulation~~ Laboratory ~~Office/Facility Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules

RISKS RELATED TO CHEMICAL HANDLING
WEAR PPE (LAB COAT, GLOVES, AND GOGGLES). SAFE HANDLING OF CHEMICALS CONTAINED IN CLOSED CONTAINERS AND STORED APPROPRIETLY ACCORDING TO THEIR LEVEL OF HAZARD, DISPOSE OF CHEMICALS IN SEPARATE CONTAINER FOR DISPOSAL.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Davidson's Seawater Fixative (includes ethanol, formaldehyde, glycerine, 3% saline and glacial acetic acid)	<1L	Spillage, contact with skin/eyes, inhalation,	B	6
Ethanol	< 1L		C	3
3% Saline	<1L		D	3

Hazard Category (known or potential)
- **A** (e.g. carcinogen/teratogen/mutagen)
- **B** (e.g. v.toxic/toxic/explosive/pyrophoric)
- **C** (e.g. harmful/irritant/corrosive/high flammable/oxidising)
- **D** (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low ~~**Medium**~~ ~~**High**~~

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :- **CHEMICALS CONTAINED IN SEALED FLASK AND PLASTIC BOTTLES**

Storage conditions and maximum duration :- **CHEMICALS STORED IN CLOSED CUPBOARDS AT AMBIENT TEMPERATURE AND FRIDGES- VENTILATED CUPBOARD**

Secondary containment (of protocol) open bench/fume hood/special (state) :- **FUME HOOD AND BENCH TOP (secondary containment : Tray)**

Disposal: autoclaving of biohazard, SU chemical disposal

Identify other control measures (circle or delete) – gloves; spillage tray; safety goggles; lab coat

Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name Emma Quinn ..... **Signature** [redacted] **date** 03/18 ..

Supervisor* Christopher Coates ..... **Signatur** [redacted] **date** 03/18 .

Activity title Hemocyanin purification..... **Base location (room no.)** 123 ..

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No. .....

Start date of activity (cannot predate signature dates)09/03/2018 .....

End date of activity (or 'on going') 30/09/2020 .....

Level of worker (delete as applicable): PhD student .....

~~UG~~,PG~~, research assistant, technician, administration, academic staff, other (state)~~

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes~~/not applicable

Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes~~/not applicable

Approval obtained for use of radioisotopes by COS ? ~~Yes~~/not applicable

Record of specialist training undertaken

Course	date
Informal training provided by Dr Bettina Walter	26/02/18

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
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See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 3	**Title:** Hemolymph purification
Associated Protocols #2.....	**Description: Slipper limpet *Crepidula fornicata* hemocyanin is extracted, centrifuged, diluted and processed to quantify protein concentration.**

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat~~ ~~Field~~ ~~Genetic Manipulation~~ Laboratory ~~Office/Facility~~ ~~Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules
Wear PPE (lab coat, gloves and goggles). Safe handling of chemicals contained in closed containers and stored appropriately according to their level of hazards, dispose of chemicals in separate container.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Tris buffer	<2ml	Skin irritant Serious eye irritant	C	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

-Low ~~Medium~~ ~~High~~

Primary containment (of product) plastic bottle

Storage conditions and maximum duration :- Store in cool place. Keep container in a dry and well ventilated place.

Secondary containment (of protocol) open bench/fume hood/special (state) :- open bench

Disposal e.g. autoclaving of biohazard, SU chemical disposal: SU Chemical Waste Store, Individual labelled container

Identify other control measures – Lab coat, gloves

Justification and controls for any work outside normal hours n/a

Emergency procedures (e.g. spillage clearance; communication methods): Soak up with inert absorbent material and dispose of as hazardous waste

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker.....Emma Quinn ..... [redacted] .....

[redacted]

Name & counter-signature of supervisor..... .....
Date.....03/18.....

Date of first reassessment: 01/10/18 Frequency of reassessments: Annually

Justification and controls for any work outside normal hours NA

Emergency procedures (e.g. spillage clearance; communication methods): **First Aid Kit available in the lab. Emergency number: 333**

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker..... [redacted] .....

Name & counter-signature of supervisor

..... [redacted] .....

Date.....03/18.....

Date of first reassessment: 01/10/18 Frequency of reassessments: Annual

Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name Emma Quinn ..... **Signature** [redacted] **date** 03/18 .....

Supervisor* Christopher Coates ..... **Signature** [redacted] **date** 03/18 .....

Activity title Making TSA+2%NaCl ..... **Base location (room no.)** 123 ..

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No .....

Start date of activity (cannot predate signature dates) 09/3/2018 .....

End date of activity (or 'on going') 30/09/2020 .....

Level of worker (delete as applicable) PhD student .....

~~UG,~~PG~~, research assistant, technician, administration, academic staff, other (state)~~

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes/~~not applicable

Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes/~~not applicable

Approval obtained for use of radioisotopes by COS ? ~~Yes/~~not applicable

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
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See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 4	**Title:** Making Tryptone Soya agar (TSA) plates with 2% NaCl
Associated Protocols #.....	**Description:** Make up TSA+2%NaCl mix. Autoclave it and pour out plates.

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat~~ ~~Field~~ ~~Genetic Manipulation~~ **Laboratory** ~~Office/Facility~~ ~~Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules
Wear PPE (lab coat, gloves and goggles). Safe handling of chemicals contained in closed containers and stored appropriately according to their level of hazards, dispose of chemicals in separate container.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
TSA	40g/L	None	D	2
Sodium Chloride	20g/L	None	D	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low ~~Medium~~ ~~High~~

Primary containment (of product) plastic bottle

Storage conditions and maximum duration :- Kept at room temperature in glass cabinet.

Secondary containment (of protocol) open bench/fume hood/special (state) :- n/a

Disposal e.g. autoclaving of biohazard, SU chemical disposal: Autoclaving of plates

Identify other control measures – Lab coat, gloves

Justification and controls for any work outside normal hours n/a

Emergency procedures (e.g. spillage clearance; communication methods): Clean up with damp cloth

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker.....[redacted]

Name & counter-signature of supervisor.....[redacted]
..... *Date*..... *03/18*.....

Date of first reassessment: 01/10/18 Frequency of reassessments: Annually

Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name: Emma Quinn ..... **Signature** [redacted] **date** 03/18

Supervisor*: Dr Christopher Coates ..... **Signature** [redacted] **3/18 date** .....

Activity title: ***Crepidula fornicata*** **sampling including haemolymph, muscle and fixed sample.**
Base location (room no.): 123
(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No. .....
Start date of activity (cannot predate signature dates): 0/3/2018 .....
End date of activity (or 'on going'): 30/09/17 .....

Level of worker (delete as applicable): PhD student .....

~~UG;~~PG~~, research assistant, technician, administration, academic staff, other (state)~~

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes~~/not applicable
Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes~~/not applicable
Approval obtained for use of radioisotopes by COS ? ~~Yes~~/not applicable

Record of specialist training undertaken

Course	date
Histology training carried out by Dr Sharon Lynch UCC	03/04/17

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
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See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 4	**Title:** Histology of Slipper limpets, *Crepidula fornicata.*
Associated Protocols *#... I*	**Description:** Fix samples in Davidson's seawater fixative for 24hr, transfer into 70% ethanol, put samples into processor containing ethanol, histoclear and paraffin wax, place in vacuum/heater before leaving to set. Use Microtome to cut specimen samples and fix to slide using Mayer's Fix. Stain slides using H&E.

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat~~ ~~Field~~ ~~Genetic Manipulation~~ Laboratory ~~Office/Facility~~ ~~Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules
Wear PPE (lab coat, gloves and goggles). Safe handling of chemicals contained in closed containers and stored appropriately according to their level of hazards, dispose of chemicals in separate container.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Davidson's Seawater Fixative (includes ethanol, formaldehyde, glycerine, 3% saline and glacial acetic acid)	<1L	Flammable, Irritant, Toxic, Hazardous to health, Corrosive	B	6
Ethanol	< 4L	Flammable, Irritant,	C	3
Histoclear	<2L	Flammable, Irritant, Hazardous to the environment	C	3
Mayer's Fix (includes albumen and glycerol)	<100ml	May cause irritation	D	2
H&E staining (Eosin, Hemaoxylin, ethanol)	<1L	Flammable, Irritant	C	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low– ~~Medium~~ ~~High~~

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :- Plastic bottle for Davidson's and Histoclear, glass Winchester for ethanol

Storage conditions and maximum duration: - Chemicals are stored in closed, vented cupboards at ambient temperature.

Secondary containment (of protocol) open bench/fume hood/special (state) :- All work is carried out in a fume hood whilst the chemicals are involved.

Disposal: SU chemical disposal

Identify other control measures (circle or delete) - nitrile gloves; protective shoes; spillage tray; lab coat, safety goggles

Justification and controls for any work outside normal hours N/A

Emergency procedures (e.g. spillage clearance; communication methods): First Aid Kit available in the lab. Emergency number: 333

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker [redacted]

Name & counter-signature of supervisor Dr Christopher Coates [redacted]

Date.....*03/18*.....

Date of first reassessment: 01/10/18 Frequency of reassessments: Annual

College of Science Risk Assessment -Boat Work

Hazard	Risk / Consequence	RISK FACTOR L	C	R	Risk Reduction Measures	NEW RISK FACTOR L	C	R
WATER SAMPLE COLLECTION								
FALLING OVERBOARD	DROWNING HYPOTHERMIA	3	3	9	CLIP ONTO SAFETY HARNESS WHEN WORKING NEAR STERN OF VESSEL. WEAR SELF INFLATING LIFEJACKETS WITH INTEGRAL HARNESS. WEAR SUITABLE CLOTHING FOR WEATHER CONDITIONS.	1	1	1
SLIPPING ON WET DECK	PERSONAL INJURY, FOOT SPRAIN, BROKEN BONES AND CUTS	2	2	4	WEAR NOT SLIP FOOTWEAR AND WHEN DECANTING BOTTLES BE AWARE OF SLIPPERY DECK.	1	2	2
PLANKTON NET DEPLOYMENT								
FALLING OVERBOARD	DROWNING HYPOTHERMIA	2	3	6	CLIP ONTO SAFETY HARNESS WHEN WORKING NEAR STERN OF VESSEL. WEAR SELF INFLATING LIFEJACKETS WITH INTEGRAL HARNESS. WEAR SUITABLE CLOTHING FOR WEATHER CONDITIONS.	1	1	1
EQUIPMENT NOT MAKE SECURE BEFORE DEPLOYMENT	LOSS OF EXPENSIVE EQUIPMENT	2	2	4	DEPLOY NET ON SKIPPERS COMMAND USING ARMS ON SIDE OF A FRAME MAKE SURE NETS ARE MADE SECURE WITH A BOWLINE KNOT ON DEPLOYMENT AND RETRIEVAL MAKE SURE NETS STAY CLEAR OF PROPELLERS	1	1	1
EQUIPMENT HAS LARGE WATER RESISTANCE WHEN RETRIEVING FROM WATER	MUSCLE STRAIN	2	2	4	ON RETRIEVAL BY HAND USE WINCH IF THERE IS TOO MUCH WATER RESISTANCE ON THE NETS	1	1	1
GENERAL SHIPS SAFETY								
FALLING OVERBOARD	DROWNING	3	4	12	MARINE LIFEJACKETS WITH AUTOMATIC TRIGGERS TO BE WORK AT ALL TIMES LIFEJACKETS ARE STORED ON VESSEL AND ISSUED TO ALL PASSENGERS AND CREW - THEY ARE SERVICED ANNUALLY	**2**	2	4

MAN OVERBOARD	DROWNING	3	4	12	THE SKIPPER WILL ADVICE ON MAN OVERBOARD PROCEDURES, LOCATION AND DEPLOYMENT OF LIFE RAFTS, LOCATION OF SAFETY LADDER AND EQUIPMENT FOR RETRIEVAL OF ANY PERSONS IN THE WATER. SAFETY HARNESSES TO BE USE IN SOME WORKING CONDITIONS. THE SKIPPER IS TO BE INFORMED IMMEDIATELY IF SOMEONE FALLS IN THE WATER	2	2	4
TRIPPING AND SNAGGING (TRIPS & SLIPS)	PERSONAL INJURY, FOOT SPRAIN, BROKEN BONES AND CUTS	2	2	4	CABLES AND ROPES SHALL BE KEPT IN A TIDY ORDER, WIRES AND ROPES WILL BE UNDER TENSION WHEN TRAWLING, ALL PASSENGERS TO KEEP CLEAR OF THESE AREAS	1	2	2
COMMUNICATION	UNKNOWN POSITION OF VESSEL IN CASE OF INCIDENTS	2	2	4	THE SKIPPER WILL CONTACT THE COLLEGE OF SCIENCE (LIZ COZENS OR ROSEMARY MUXWORTHY - IN TEACHING ADMIN) WITH NAMES OF PERSONS AT SEA, DESTINATION, AND APPROXIMATE START AND FINISH TIMES. DURING INCIDENTS THE SKIPPER WILL BE INFORMED IMMEDIATELY WHOM IF NECESSARY WILL INFORM THE COASTGUARD USING VHF CHANNEL 16	1	1	2
ADVERSE WEATHER	SLIPPING AND FALLING	2	2	4	THE SKIPPER WILL ONLY PUT TO SEA WHEN CONDITIONS ARE FAVOURABLE WHEN WORKING ON QUAYSIDE OR AT SEA TAKE CARE WHEN WET OR ICY IF IN DOUBT DO NOT APPROACH WATERS EDGE. WEAR SUITABLE NON SLIP FOOTWEAR.	1	1	2
POTENTIAL HARMFUL SPECIES WEAVER FISH, JELLY FISH ETC	BITE, STINGS AND VENOM	3	2	6	ALWAYS BE AWARE OF HARMFUL SPECIES WHICH MAY BE ENCOUNTERED, SAFE HANDLING PROTOCOLS SHALL ALWAYS BE ADOPTED. GLOVES SHALL BE WORN SORTING THROUGH TRAWL CATCH. INFORM OTHERS IF DANGEROUS SPECIES ARE OBSERVED	1	1	1

FIRE	BURNS AND DAMAGE TO VESSEL	1	4	4	CREW WILL LOCATE ENGINE ON FIRE, CUT OFF FUEL SUPPLY TO ENGINE, ACTIVATE FIRE EXTINGUISHERS. IN CASE OF A COOKER FIRE ISOLATE GAS SUPPLY AND TREAT AS NORMAL COOKER FIRE USING FIRE BLANKET AND EXTINGUISHER	**1**	2	2
SEA SICKNESS	FEELING UNWELL. DEHYDRATION FROM VOMITING	3	2	6	INFORM THE SKIPPER IF YOU USUALLY SUFFER FROM SEA SICKNESS, IF YOU DO BRING NON- DROWSY SEA SICKNESS TABLETS, TAKE FIRST TABLET BEFORE LEAVING THE BERTH. EATING GINGER BASED PRODUCTS CAN HELP PREVENT NAUSEA. DRINK WATER ONLY.	**2**	2	4
HANDLING DANGEROUS SUBSTANCES (ETHANOL IS THE ONLY CHEMICAL COMMONLY USED ON BOARD)	HIGHLY INFLAMMABLE, EYE SKIN CONTACT, INGESTION	2	2	4	EYE SKIN CONTACT - RINSE IMMEDIATELY WIT PLENTY OF WATER INGESTION - DO NOT INDUCE VOMITING, OBTAIN MEDICAL ATTENTION	2	2	4
LIFTING EQUIPMENT	DAMAGE TO LIMBS	2	4	8	ALL LIFTING EQUIPMENT OPERATIONS SHALL BE SUBJECT TO A THROUGH ANNUAL EXAMINATION UNDER 'LIFTING OPERATIONS AND LIFTING EQUIPMENT REGULATIONS 1998' ANY REPORTED DEFECTS SHALL BE REPORTED TO THE SKIPPER IMMEDIATELY AND THE DEFECTED EQUIPMENT TAKEN OUT OF ACTION.	**2**	2	4
SHARP OBJECT	CUTS	2	2	4	APPROPRIATE PPE TO BE WORN - GLOVES. MEDICAL ATTENTION WILL BE SOUGHT IN CASE OF INFECTION.	**1**	1	1

Name	Emma Quinn	Supervisor's Name	Dr Christopher Coates
Signature	[redacted]	Supervisor's Signature	[redacted]
Date	01/03/2018	Date	01/ 03/18

Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name: Emma Quinn .....**Signature** [redacted] **date** 14/06/19

Line manager*: Christopher Coates .....**Signature** [redacted] **date** 14/06/19

Activity title: PCR, amplicons purification and agarose electrophoresis
Base location (room no.): 131A
(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No.....
Start date of activity (cannot predate signature dates): 14/06/19.....
End date of activity (or 'on going'): Ongoing .....

Level of worker (delete as applicable): Postgraduate Student (BlueFish Innovation PhD bursary)

~~UG,PG, research assistant, technician, administration, academic staff,~~ other (state)

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes~~/not applicable
Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes~~/not applicable
Approval obtained for use of radioisotopes by COS ? ~~Yes~~/not applicable

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
2						12					
3						13					
4						14					
5						15					
6						16					
7						17					
8						18					
9						19					
10						20					

See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 1	**Title:** PCR
Associated Protocols #**.....**	**Description:** Amplification of DNA fragments using PCR

Location:

circle which **Bioscience** and Geography Local Rules apply –

~~Boat Field Genetic Manipulation~~ **Laboratory** ~~Office/Facility Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules
RISKS RELATED TO CHEMICAL HANDLING
WEAR LAB COAT AND GLOVES. SAFE HANDLING OF CHEMICALS CONTAINED IN CLOSED CONTAINERS AND STORED APPROPRIETLY ACCORDING TO THEIR LEVEL OF HAZARD, DISPOSE OF CHEMICALS IN SEPARATE CONTAINER FOR DISPOSAL.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
PCR master mix	< 1 ml		D	1
Primers	< 1 ml		D	1
DNA	< 1 ml		D	1

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low **Medium** **High**

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :- **CHEMICALS CONTAINED IN SEALED FLASK AND PLASTIC BOTTLES**

Storage conditions and maximum duration :- **CHEMICALS STORED IN CLOSED CUPBOARDS AT AMBIENT TEMPERATURE AND FRIDGES- VENTILATED CUPBOARD**

Secondary containment (of protocol) open bench/fume hood/special (state) :- **FUME HOOD AND BENCH TOP (secondary containment : Tray)**

Disposal: autoclaving of biohazard, SU chemical disposal

Identify other control measures (circle or delete) – gloves; lab coat

Justification and controls for any work outside normal hours NA

Emergency procedures (e.g. spillage clearance; communication methods): **First Aid Kit available in the lab. Emergency number: 333**

Supervision/training for worker (circle)

None required **Already trained** Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker Emma Quinn .....[redacted].....
.....

Name & counter-signature of supervisor Christopher Coates

[redacted]
....

Date.....

Date of first reassessment Frequency of reassessments:

Protocol # 2	Title: Electrophoresis
Associated Protocols #.....	**Description:** Separation (electrophoresis) and visualization of amplicons and DNA in agarose gels

Location:

circle which **Bioscience** and Geography Local Rules apply –

~~Boat Field Genetic Manipulation~~ Laboratory ~~Office/Facility Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules
RISKS RELATED TO CHEMICAL HANDLING
WEAR LAB COAT AND GLOVES SAFE HANDLING OF CHEMICALS CONTAINED IN CLOSED CONTAINERS AND STORED APPROPRIETLY ACCORDING TO THEIR LEVEL OF HAZARD, DISPOSE OF CHEMICALS IN SEPARATE CONTAINER FOR DISPOSAL.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Agarose	100 g		D	2
Green Safe Premium	< 1 ml		D	1
NZY Ladder V	< 1 ml		D	1
1X TBE Buffer	100ml		C	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low **Medium** **High**

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :- **CHEMICALS CONTAINED IN SEALED FLASK AND PLASTIC BOTTLES**

Storage conditions and maximum duration :- **CHEMICALS STORED IN CLOSED CUPBOARDS AT AMBIENT TEMPERATURE AND FRIDGES- VENTILATED CUPBOARD**

Secondary containment (of protocol) open bench/fume hood/special (state) :- **FUME HOOD AND BENCH TOP**

Disposal: autoclaving of biohazard, SU chemical disposal

Identify other control measures (circle or delete) – gloves; spillage tray; safety goggles; lab coat

Justification and controls for any work outside normal hours NA

Emergency procedures (e.g. spillage clearance; communication methods): **First Aid Kit available in the lab. Emergency number: 333**

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker Emma Quinn .....[redacted].....

Name & counter-signature of supervisor Christopher Coates[redacted]
.....*Date*.....06/19.....

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 3	**Title:** Nucleic acids QUANTIFICATION
Associated Protocols #.....	**Description:** Quantification of DNA using Qubit3 Fluorometer

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat Field Genetic Manipulation~~ Laboratory ~~Office/Facility Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules

RISKS RELATED TO CHEMICAL HANDLING
WEAR LAB COAT AND GLOVES. SAFE HANDLING OF CHEMICALS CONTAINED IN CLOSED CONTAINERS AND STORED APPROPRIETLY ACCORDING TO THEIR LEVEL OF HAZARD, DISPOSE OF CHEMICALS IN SEPARATE CONTAINER FOR DISPOSAL.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Qubit dsDNA HS Reagent (Component A)	1 ml		D	2
Qubit dsDNA HS Buffer (Component B)	250 mL		D	3
Qubit dsDNA HS Standard (Component C)	5 mL		D	2
Qubit dsDNA HS Standard (Component D)	5 mL		D	2
Qubit RNA HS Reagent (Component A)	250 µL		D	1
Qubit RNA HS Buffer (Component B)	50 mL		D	2
Qubit RNA HS Standard (Component C)	1 mL		D	2
Qubit RNA HS Standard (Component D)	4 × 250 µL		D	2

Hazard Category (known or potential)	**Exposure Potential** Circle the **highest Exposure Score** above. Use this to calculate the exposure
A (e.g. carcinogen/teratogen/mutagen)	

B (e.g. v.toxic/toxic/explosive/pyrophoric) **C** (e.g. harmful/irritant/corrosive/high flammable/oxidising) **D** (e.g. non classified)	potential for the entire protocol (see handbook). Indicate this value below. **Low** **Medium** **High**
Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :- **CHEMICALS CONTAINED IN SEALED FLASK AND PLASTIC BOTTLES** **Storage conditions and maximum duration** :- **CHEMICALS STORED IN CLOSED CUPBOARDS AT AMBIENT TEMPERATURE AND FRIDGES- VENTILATED CUPBOARD**	
Secondary containment (of protocol) open bench/fume hood/special (state) :- **FUME HOOD AND BENCH TOP (secondary containment : Tray)**	
Disposal: autoclaving of biohazard, SU chemical disposal	
Identify other control measures (circle or delete) – gloves; spillage tray; safety goggles; lab coat	
Justification and controls for any work outside normal hours NA	
Emergency procedures (e.g. spillage clearance; communication methods): **First Aid Kit available in the lab. Emergency number: 333**	
Supervision/training for worker (circle) None required Already trained Training required Supervised always	
Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures. *Name & signature of worker* Emma Quinn..... *Name & counter-signature of supervisor* Christopher Coates..... .....*Date*.....*06/19*.....	
Date of first reassessment	Frequency of reassessments:

Risk Assessment for Teaching, Administration and Research Activities
Swansea University; College of Science

Name: Emma Quinn ..... **Signature** [redacted] **date** 14/06/19

Line manager*: Christopher Coates ..... **Signature** [redacted] **date** 14/06/19

Activity title: PCR, amplicons purification and agarose electrophoresis
Base location (room no.): 131A
(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)
University Activity Serial # (enter Employee No. or STUREC No. .....
Start date of activity (cannot predate signature dates): 14/06/19 .....
End date of activity (or 'on going'): Ongoing .....
Level of worker (delete as applicable): Postgraduate Student (BlueFish Innovation PhD bursary)

~~UG,PG, research assistant, technician, administration, academic staff,~~ other (state)

Approval obtained for Gene Manipulation Safety Assessment by SU ? ~~Yes/~~not applicable
Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes/~~not applicable
Approval obtained for use of radioisotopes by COS ? ~~Yes/~~not applicable

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-	Hazard	Secondary	Exposure		1st date	Frequency of re-	Hazard	Secondary	Exposure
1						11					
2						12					
3						13					
4						14					
5						15					
6						16					
7						17					
8						18					
9						19					
10						20					

See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form
(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 2	**Title:** DNA Extraction – SIGMA GenElute Blood Genomic DNA Kit
Associated Protocols #.....	**Description:** Extraction of DNA from *Crepidula fornicata* using a SIGMA GenElute Blood Genomic DNA Kit

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat~~ ~~Field~~ ~~Genetic Manipulation~~ Laboratory ~~Office/Facility~~ ~~Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules

RISKS RELATED TO CHEMICAL HANDLING
WEAR LAB COAT AND GLOVES. SAFE HANDLING OF CHEMICALS CONTAINED IN CLOSED CONTAINERS AND STORED APPROPRIETLY ACCORDING TO THEIR LEVEL OF HAZARD, DISPOSE OF CHEMICALS IN SEPARATE CONTAINER FOR DISPOSAL.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Resuspension Solution	100 mL		D	2
Lysis Solution C	90 mL		D	3
Wash Solution Concentrate	90 mL		C	2
Elution Solution	180 mL	Causes skin irritation/Causes serious eye irritation		
Column Preparation Solution	225 mL	May cause allergy or asthma symptoms or breathing difficulties if inhaled.	C	2
Proteinase K	200 mg			
RNase A solution	8 mL			

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low **Medium** **High**

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :- **CHEMICALS CONTAINED IN SEALED FLASK AND PLASTIC BOTTLES**

Storage conditions and maximum duration :- **CHEMICALS STORED IN CLOSED CUPBOARDS AT AMBIENT TEMPERATURE AND FRIDGES- VENTILATED CUPBOARD**

Secondary containment (of protocol) open bench/fume hood/special (state) :- **FUME HOOD AND BENCH TOP (secondary containment : Tray)**

Disposal: autoclaving of biohazard, SU chemical disposal

Identify other control measures (circle or delete) – gloves; lab coat

Justification and controls for any work outside normal hours NA

Emergency procedures (e.g. spillage clearance, communication methods): **First Aid Kit available in the lab. Emergency number: 333**

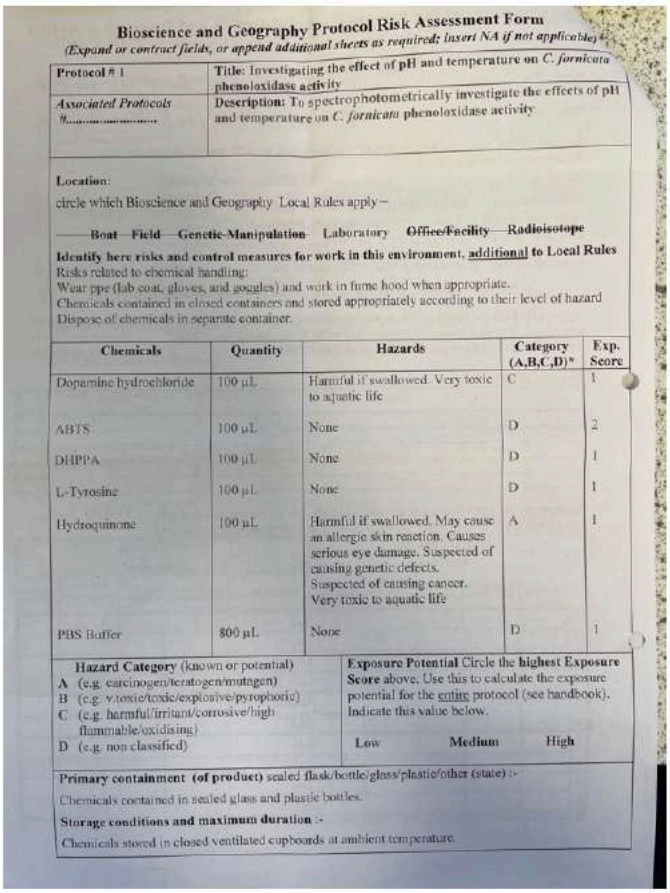
Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these ris[redacted]

Name & signature of worker Emma Quinn.....[redacted]

Name & counter-signature of supervisor Christopher Coates.....[redacted]
.....Date..... 14/06/19
.....



Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol # 1	**Title:** Investigating the effect of **pH** and temperature on *C. fornicata* **phenoloxidase activity**
Associated Protocols #	**Description:** To spectrophotometrically investigate the effects of pH and temperature on *C. fornicata* **phenoloxidase activity**

Location:

circle which Bioscience and Geography Local Rules apply –

~~Boat~~ ~~Field~~ ~~Genetic Manipulation~~ Laboratory ~~Office/Facility~~ ~~Radioisotope~~

Identify here risks and control measures for work in this environment, additional to Local Rules

Risks related to chemical handling:
Wear ppe (lab coat, gloves, and goggles) and work in fume hood when appropriate.
Chemicals contained in closed containers and stored appropriately according to their level of hazard
Dispose of chemicals in separate container.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Dopamine hydrochloride	100 µL	Harmful if swallowed. Very toxic to aquatic life	C	1
ABTS	100 µL	None	D	2
DHPPA	100 µL	None	D	1
L-Tyrosine	100 µL	None	D	1
Hydroquinone	100 µL	Harmful if swallowed. May cause an allergic skin reaction. Causes serious eye damage. Suspected of causing genetic defects. Suspected of causing cancer. Very toxic to aquatic life	A	1
PBS Buffer	800 µL	None	D	1

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

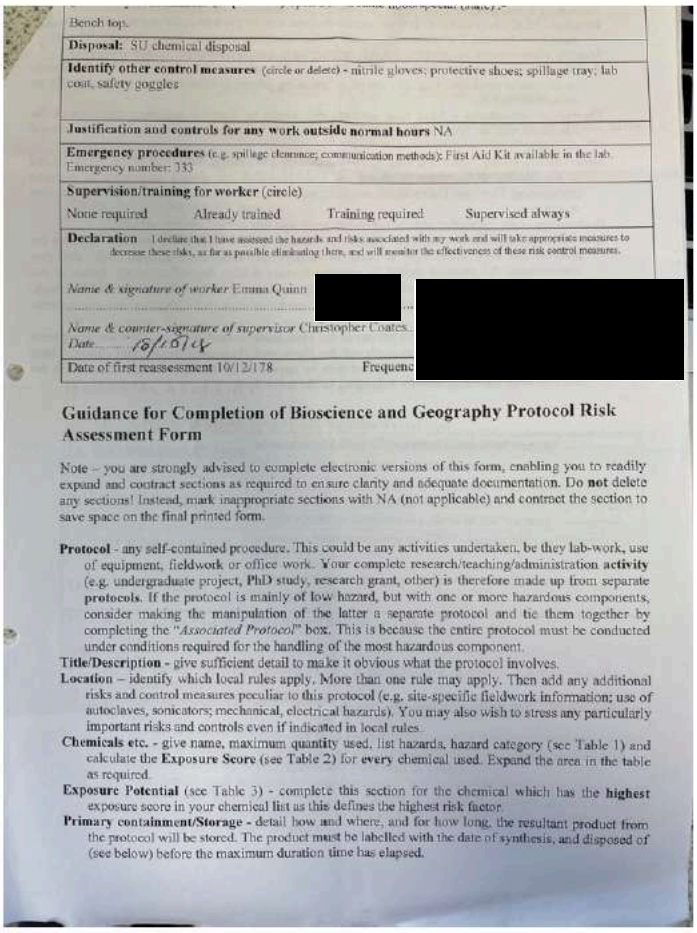
Low **Medium** **High**

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :-

Chemicals contained in sealed glass and plastic bottles.

Storage conditions and maximum duration :-

Chemicals stored in closed ventilated cupboards at ambient temperature.



Bench top.

Disposal: SU chemical disposal

Identify other control measures (circle or delete) - nitrile gloves; protective shoes; spillage tray; lab coat, safety goggles

Justification and controls for any work outside normal hours NA

Emergency procedures (e.g. spillage clearance; communication methods): First Aid Kit available in the lab. Emergency number: 333

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker Emma Quinn [redacted]

Name & counter-signature of supervisor Christopher Coates [redacted]

Date..... 16/10/18

Date of first reassessment 10/12/178 Frequenc [redacted]

Guidance for Completion of Bioscience and Geography Protocol Risk Assessment Form

Note – you are strongly advised to complete electronic versions of this form, enabling you to readily expand and contract sections as required to ensure clarity and adequate documentation. Do **not** delete any sections! Instead, mark inappropriate sections with NA (not applicable) and contract the section to save space on the final printed form.

Protocol - any self-contained procedure. This could be any activities undertaken, be they lab-work, use of equipment, fieldwork or office work. Your complete research/teaching/administration **activity** (e.g. undergraduate project, PhD study, research grant, other) is therefore made up from separate **protocols**. If the protocol is mainly of low hazard, but with one or more hazardous components, consider making the manipulation of the latter a separate protocol and tie them together by completing the "*Associated Protocol*" box. This is because the entire protocol must be conducted under conditions required for the handling of the most hazardous component.

Title/Description - give sufficient detail to make it obvious what the protocol involves.

Location – identify which local rules apply. More than one rule may apply. Then add any additional risks and control measures peculiar to this protocol (e.g. site-specific fieldwork information; use of autoclaves, sonicators; mechanical, electrical hazards). You may also wish to stress any particularly important risks and controls even if indicated in local rules.

Chemicals etc. - give name, maximum quantity used, list hazards, hazard category (see Table 1) and calculate the **Exposure Score** (see Table 2) for **every** chemical used. Expand the area in the table as required.

Exposure Potential (see Table 3) - complete this section for the chemical which has the **highest** exposure score in your chemical list as this defines the highest risk factor.

Primary containment/Storage - detail how and where, and for how long, the resultant product from the protocol will be stored. The product must be labelled with the date of synthesis, and disposed of (see below) before the maximum duration time has elapsed.