DIGENEAN PARASITES OF SHORE CRABS, *CARCINUS MAENAS*

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Summary

This study reports on the prevalence, intensity, and species identification of digenean trematode infections in shore crabs (*Carcinus maenas*) found at two intertidal survey sites (Mumbles Head and Oxwich Bay) in the Bristol Channel, U.K. between February, and August 2024. Crabs were assessed for the presence and intensity of trematode infections by the microscopic examination of the hepatopancreas. The metacercariae species identification resulted from the combined use of morphological and PCR-based methods, specifically the 28S rDNA gene. Encysted metacercariae presented in two distinct forms, spherical and asymmetrical, which were shown to be *Microphallus similis* and *M. primas*, respectively. A high prevalence and intensity of *M. similis* metacercariae was found in shore crab populations from both locations throughout the duration of the study. Conversely, *M. primas* metacercariae were only identified in a limited number of crabs and at low infection intensities during June and August sampling. No cellular host immune responses were observed. Binomial logistic regression models revealed size and colouration to be significant factors in trematode presence at Oxwich Bay only. Additionally, larger/older shore crabs were found to harbour more metacercarial cysts, with larger metacercariae observed in crabs from Mumbles Head. Overall, the findings of this study highlight the widespread prevalence of *M. similis* infections in shore crab populations at both intertidal survey sites, with notable size and location-specific variations in infection intensity. Additionally, the results underscore the importance of conducting a long-term study to better understand the seasonal dynamics of *M. similis* and *M. primas* infections in shore crabs.

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Table of contents

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List of figures

Chapter 1

Chapter 3

Appendix

List of tables

Chapter 1

Chapter 3

Appendix

Chapter One

General Introduction

Note: This thesis examines the interaction between the host (the European shore crab, *Carcinus maenas*) and trematode parasites belonging to the Microphallidae. Therefore, this General Introduction first reviews the general biology and known diseases of the host. Secondly, it reviews parasites belonging to the Microphallidae, their distribution, hosts and general biology.

1.1 The host

1.1.1 Life history and distribution

The shore crab (*Carcinus maenas*), also known as the European shore or green crab outside of its native home range, is a decapod crustacean from the Carcinidae family, within the phylum Arthropoda. Shore crabs are characterised by a pentagonal shaped carapace with distinctly serrated margins and a granular texture, typically adorned with mottled patterns of green, brown, yellow hues on their ventral surface (Crothers 1980; Yamada 2001; Figure 1).

Figure 1. Photograph displaying the dorsal features of a shore crab (*Carcinus maenas*). Image from:https://www.marinepests.gov.au/pests/identify/european-green-shore-crab

Shore crabs are renowned for their formidable adaptability to a wide range of environmental conditions (Young and Elliott 2019). This has facilitated their widespread distribution across the globe (Ens et al. 2022; Figure 2). Native to the north-eastern Atlantic Ocean, its indigenous range extends from Norway to Iceland, down to Mauritania on the northwest coast of Africa (Ens et al. 2022). Within this native range, this species is prevalent along the coastlines of the British Isles, the North Sea, the Baltic Sea, and the Atlantic coasts of France, Spain, and Portugal (Ens et al. 2022).

Beyond its native range, *C. maenas* has established populations in numerous non-native regions, becoming a notorious invasive non-native species (INNS) (Frizzera et al. 2021; Ens et al. 2022). In North America, it was first recorded in the early 19th century and has since spread along both the Atlantic and Pacific coasts, from Newfoundland to South Carolina and from Alaska to

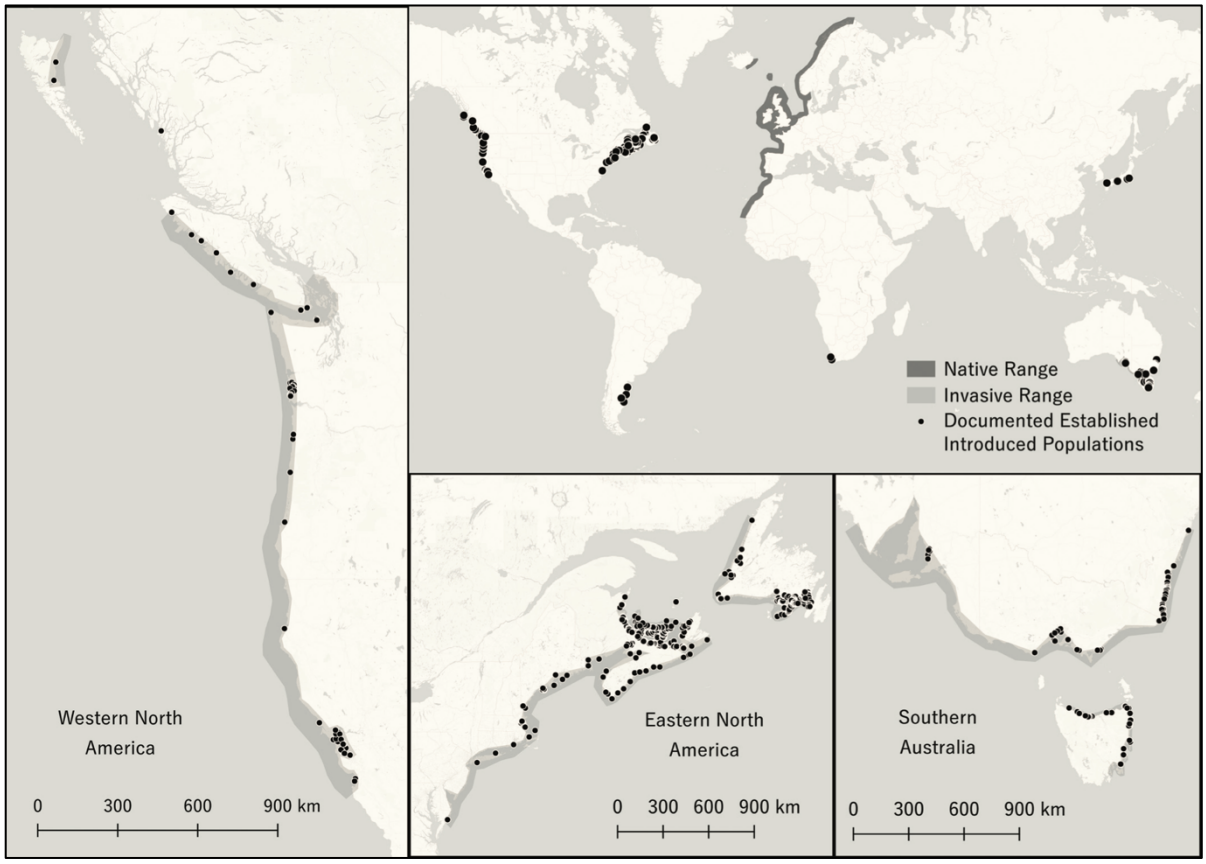

Figure 2. Global distribution of *Carcinus maenas* (green crab). The map highlights the species' native range (dark grey), invasive range (light grey), and regions with documented established populations (dotted areas). Figure and legend from Ens et al. (2022).

California (Ens et al. 2022; Figure 2). It is also present in South America, Australia, South Africa, and parts of Asia, including Japan (Ens et al. 2022)

1.1.2 Habitat

Carcinus maenas are found across diverse coastal and estuarine habitats, including rocky shores, sandy beaches, mudflats, and salt marshes (Yamada 2001; Young and Elliott 2019). These crabs exhibit wide tolerances to a range of salinities, 35 ppt in full seawater to as low as 4 ppt in brackish waters and can survive temperatures from near freezing to 35 °C (Broekhuysen 1937), with optimal conditions between 10 °C and 25 °C (Klassen and Locke 2007). Rocky intertidal zones, lying between the high and low tide marks, provide an abundance of crevices, tide pools, and vegetation that offers protection from predators and environmental challenges such as wave action and desiccation at low tide (Yamada 2001). This structurally complex habitat supports a diverse prey

community that includes molluscs, small crustaceans, and polychaetes, essential to the generalist diet of shore crabs (Cordone et al. 2023; Crothers 1980).

1.1.3 Reproduction

In native European shore crabs, reproduction takes place in the warmer months, between June and October, with a pronounced peak in August (Broekhuysen 1937). In this species, gonadal development typically occurs once the carapace width measures *ca*. 30 – 35 mm (Broekhuysen 1937; Reid et al. 1994). Once sexual maturity is reached, a male shore crab will locate a receptive female in shallower waters, near the shoreline, via the release of chemical signals from the female (Klassen and Locke 2007). Competition between males occurs throughout the mating process, with mate success favouring larger male shore crabs (Berrill 1982; Reid et al. 1994). Successful males engage the receptive female in a position known as amplexus, in which they will stay attached for up to three days (Yamada 2001). After female ecdysis, the male transfers spermatophores directly into the female's seminal receptacles (Hartnoll 1969). These spermatophores are stored in a specialized sac called the spermathecae, which allows the female to fertilize eggs up to five months after mating, thus maximizing her reproductive output (Lyons et al. 2012).

Across all global populations of *C. maenas*, an ovigerous female can produce one or two clutches annually (Klassen and Locke 2007). Research conducted in Wales, UK, in the 1960s demonstrated that ovigerous females can be observed year-round, with a pronounced peak occurring between March and April (Naylor 1962). The frequency of clutches and egg-bearing capacity of female *C. maenas* is influenced by a multitude of factors, including size of the female, temperature, and resource availability (Yamada 2001). Under optimal conditions, Broekhuysen (1937) estimates that a moderately sized female (CW 46 mm) female shore crab can produce up to 185,000 eggs per clutch. In native range *C. maenas* populations, dispersal of eggs occurs in shallow waters, typically between February and July (Queiroga et al. 1994; Baeta et al. 2005). Research indicates that the timing and dispersal of eggs is linked to optimal environmental conditions such as temperature, salinity, and food availability, ensuring that larvae develop under favourable conditions, thereby maximising survival (Broekhuysen 1937; Best et al. 2017).

1.1.4 Colouration

Typically, the sternite (ventral) surface of shore crabs (*Carcinus maenas*) appear as red or green, with yellow morphs classed as an intermediate colouration (Yamada 2001; Young et al. 2017). Colour variation in shore crabs is associated with the process of ecdysis; newly moulted crabs

typically exhibit a green colouration, which shifts to red throughout the intermoult phase (Himes et al. 2017). Conspecifics inhabiting different environments exhibit varying degrees of colour variation, with this environmental association particularly evident in homogeneous environments, where distinct colouration is not a requirement for adequate camouflage (Stevens et al. 2014a). In laboratory conditions, juvenile *C. maenas* (CW <30mm) have demonstrated phenotypic plasticity by adjusting the lightness and darkness of their carapace colour to match with their background environment (Stevens et al. 2014b). Beyond camouflage, physiological differences have also been observed between different colour morphs (Styrishave et al. 2004; Himes et al. 2017). Styrishave et al (2004) noted that during reproduction, larger male shore crabs (CW >60mm) enter a growth phase, termed anecdysis, which results in darkened, thicker carapace, and a larger master chelae which provides a competitive mating advantage over their green counterparts (Yamada 2001; Young and Elliott 2019). Red phase *C. maenas* display a reduced ability to tolerate low salinity conditions compared to green-coloured crabs, and therefore constrained to subtidal salinity conditions (McGaw and Naylor 1992; Himes et al. 2017).

1.1.5 Migration and invasion

The shore crab, originally indigenous to the coasts of Europe and North Africa, is currently ranked as the 18th most invasive non-native invasive species by the Invasive Species Specialist Group of the World Conservation Union (IUCN) (Global Invasive Species Database, 2024). The earliest recorded instance of invasiveness dates to the early 19th century along the east coasts of North America (Ens et al. 2022; Figure 3). Historically, the transoceanic dispersal of *C. maenas* has been primarily linked to the unintentional transport of larvae in the ballast water of wooden ships (Carlton and Cohen 2003). However, over the past century, the intensification of global trade and shipping activities has expanded the potential mechanisms for *C. maenas* dispersal (Carlton & Cohen 2003), facilitating the establishment of populations in geographically distant regions such as Australia, Brazil, Pakistan, Argentina, and South Korea (Ens et al. 2022; Figures 2 & 3). Additionally, *Carcinus aestuarii,* a closely related and morphologically similar species, has also been recorded beyond its native home range of the Mediterranean Sea (Geller et al. 1997; Carlton and Cohen 2003). For instance, in Japan, the distribution of both invasive *C. maenas* and *C. aestuarii* is analogous to their distribution in the north-eastern Atlantic, with *C. maenas* in the northern regions and *C. aestuarii* in southern locales (Carlton and Cohen 2003). In transitional zones, where these species coexist, morphometric (Clark et al. 2001) and genetic analysis (Geller et al. 1997) has reported a lack of complete genetic separation, indicating the formation of hybrid populations with invasive potential (Carlton and Cohen 2003; Frizzera et al. 2021).

As an invasive non-native species (INNS) the introduction of *C. maenas* raises significant ecological concerns, particularly due to the shore crab's potential to act as a vector for symbionts (Bojko et al. 2021). These symbionts/parasites may be acquired from three locations: the invasive host's native range, an intermediate point along the invasion pathway, or the new environment in which invasive host has established itself (Frizzera et al. 2021). The translocation of non-native parasites via INNS can increase the risk of disease emergence, potentially resulting in detrimental consequences for the local ecosystem (Prenter et al. 2004; Peeler et al. 2011). Such events are primarily driven by parasite host switching, a phenomenon that can occur in two ways:

1. When a non-native parasite spreads from an invasive species to a native species (termed spillover effect), it can have severe effects on the native population due to the new host's lack of immunity to the invasive parasite (Kelly et al. 2009).
2. When native hosts spread their parasites to the invasive species (termed spillback effect), which can alter host-parasite dynamics, and lead to increased transmission rates within the native species population (Kelly et al. 2009).

The severity of parasite transmission between an INNS and a native host depends on several factors: the invasive host's parasite load, the native host's co-evolutionary history with the invasive parasite, the parasite's life cycle characteristics, and the duration since the parasite's introduction to the local ecosystem (Prenter et al. 2004; Blakeslee et al. 2013; Frizzera et al. 2021). Many of these factors apply to the co-introduction of parasites by invasive shore crabs. As an INNS, shore crabs harbour a diverse array of parasites, each with varying levels of life cycle complexity (Blakeslee et al. 2015; Rowley et al. 2020; Bojko et al. 2021; Li et al. 2021). Additionally, the geographical separation of invasive shore crab populations and the native hosts they encounter limits the co-evolutionary history between the introduced parasites of the invasive shore crabs and these potential new hosts (Prenter et al. 2004; Peeler et al. 2011; Ens et al. 2022). This separation can be viewed as an advantage for *C. maenas* in terms of 'parasite escape'. Specifically, non-native invasive hosts like *C. maenas* in some regions at least, can evade between 67% and 100% of the parasite species that would typically infect them in their native home range (Blakeslee et al. 2013). It has been shown that a reduction in parasite burden can also enhance an invasive species' fitness by promoting greater energy allocation towards growth, reproduction, and survival, thereby increasing their establishment success in novel environments (Blakeslee et al., 2013; Colautti et al., 2004; Torchin & Mitchell, 2004).

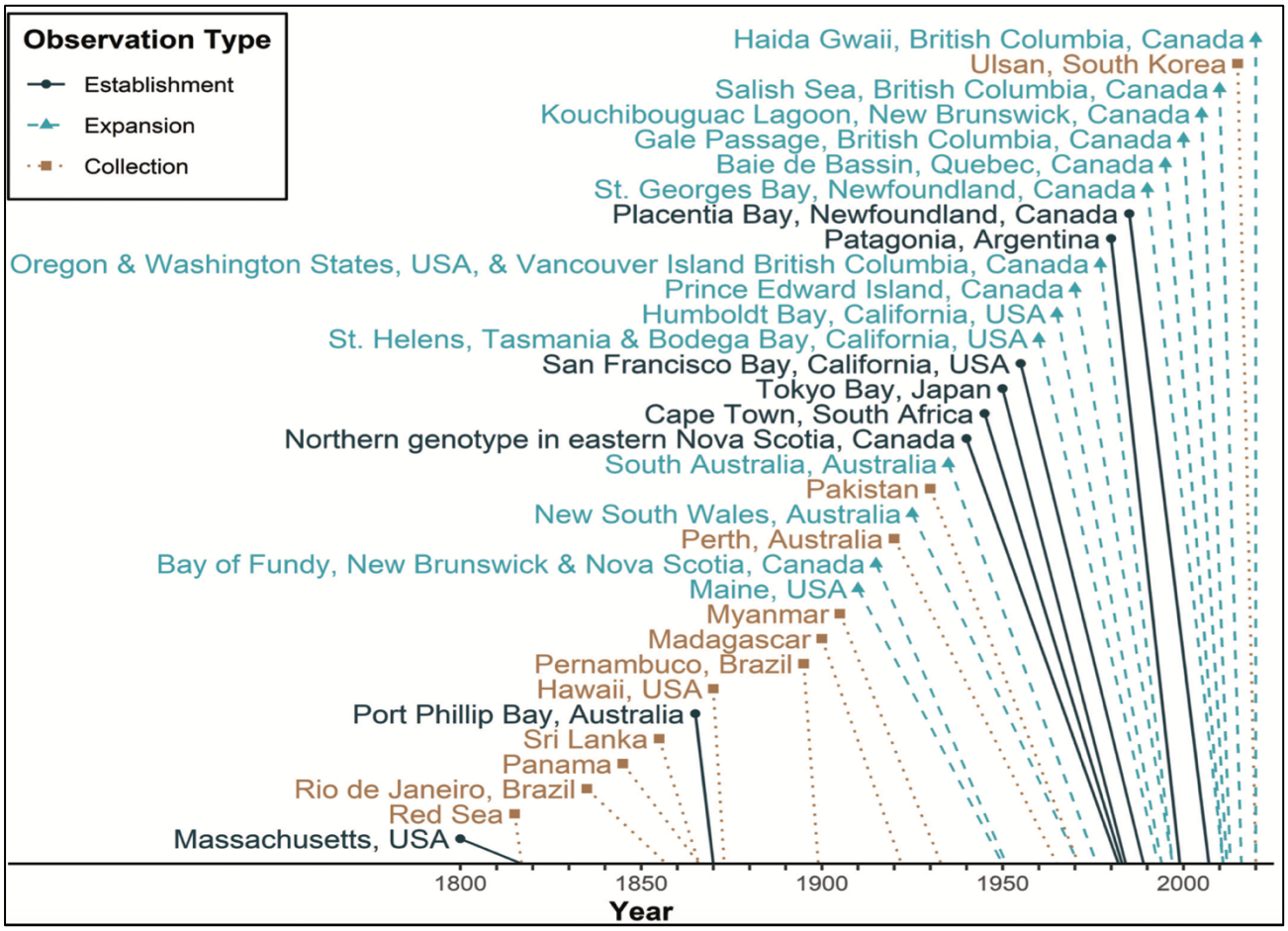

Figure 3. Timeline from the expansion of shore crabs (*Carcinus maenas*) beyond its native home range. establishment: observation of a breeding population, expansion: range expansion from location of establishment, collection: where *C.maenas* were collected, but no breeding population recorded. Figure and legend from Ens et al. (2022).

1.1.6 Disease of shore crabs – a brief overview

Shore crabs are probably the most extensively studied decapod crustacean within the field of marine pathobiology (Table 1). They are a host to numerous disease-causing agents including viruses, bacteria, fungi, microsporidians, nematodes, acanthocephalans, digeneans, and parasitic barnacles (e.g. *Sacculina carcini*) (Table 1, Figure 4). Although many of these disease-causing agents result in mortality, some, such as microphallids, utilise *C. maenas* as an intermediate host to complete their life cycle (Galaktionov & Dobrovolskij, 2013; Pina et al., 2011b; Saville & Irwin, 1991). For these commensal digeneans, their free-living larval stages enter the crab and form encysted metacercaria within tissue, which are not excised until the crab is ingested by the definitive host (McCarthy et al., 2002; Morley, 2020; Stunkard, 1957; Table 1, Figure 4).

The parasitic barnacle (*S. carcini*) is termed a "parasitic castrator" due to its capacity to cause the development of female sex characteristics in male *C. maenas* and atrophy of the gonadal tissue (Kristensen et al. 2012; Rowley et al. 2020). Furthermore, infected crabs exhibit behavioural changes in response to the developing externa that mimics the behaviour of egg-bearing female *C. maenas* (Thresher et al. 2000; Larsen et al. 2013). The parasitic dinoflagellate of the genus *Hematodinium* is of concern to commercial crab fisheries due to infection resulting in bitter meat (at least in some species) (Meyers et al. 1996; Shields et al. 2005; Table 1; Figure 4a-c). In Swansea, Wales, *Hematodinium*-positive shore crabs were surveyed to investigate where infection drives the contraction of collateral diseases (Davies et al. 2022). However, it was concluded that rather than generalised immune suppression, *Hematodinium* sp. evades immune activation, therefore there is no increased susceptibility to co-infections (Rowley et al. 2015). Haplosporidians are a group of protistan parasites known for infecting a wide range of marine invertebrates (Arzul and Carnegie 2015). Recent reports have identified two novel species of haplosporidian in shore crabs surveyed from Swansea, Wales, with these parasites displaying distinct morphologies of monokaryotic and dikaryotic unicellular stages (Davies et al. 2020a; Table 1; Figure 4e).

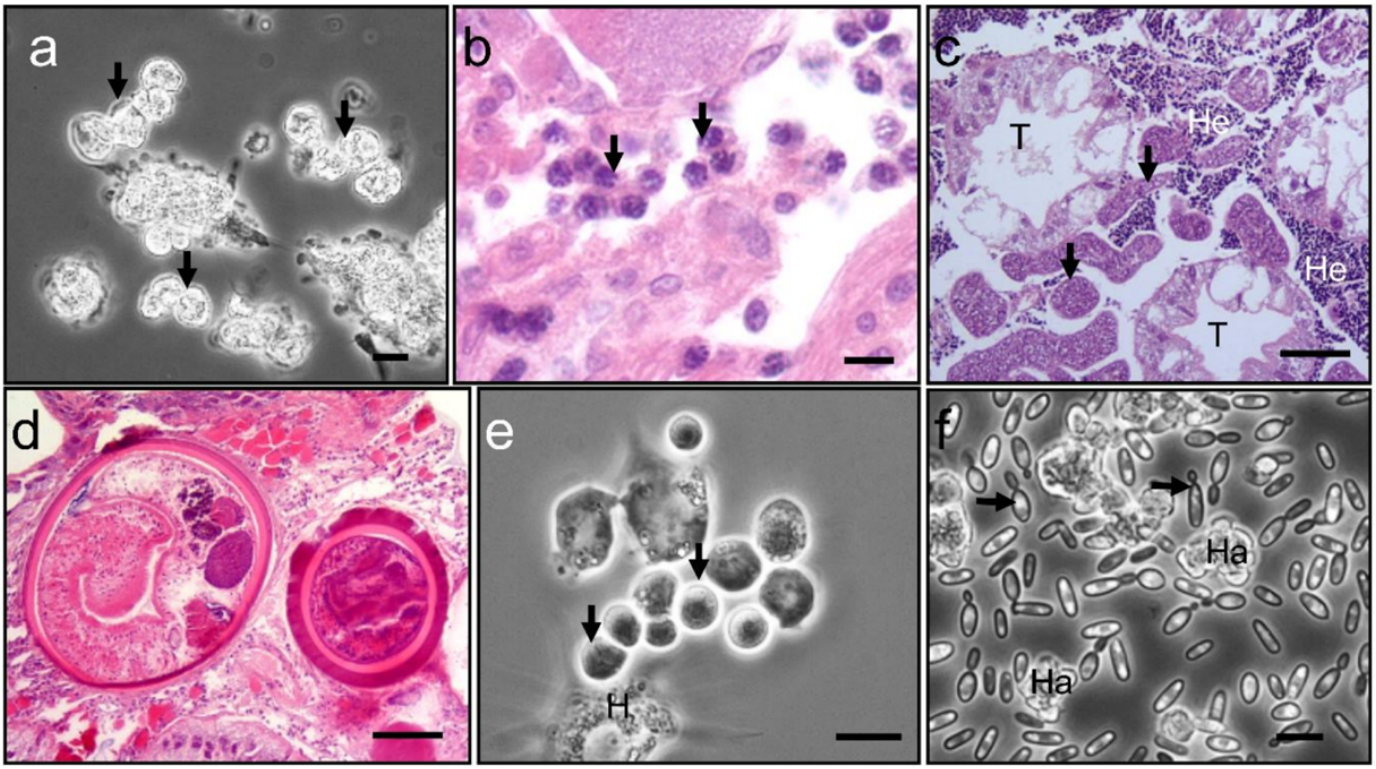

Figure 4. Diseases of shore crabs (*Carcinus maenas*)(**a**, **b**) Dinoflagellate parasite, *Hematodinium* (arrows), found in the haemolymph (a) and gonadal tissue (**b**). Scale bars = 10 µm. (**c**) Co-infected crab with the parasitic barnacle, *Sacculina carcini* (arrowheads) and *Hematodinium* (He), in the hepatopancreas. Hepatopancreatic tubules (T). Scale bar = 100 µm. (**d**) Encysted digenean trematode parasites in the hepatopancreas. Scale bar = 100 µm. (**e**) *Haplosporidium carcini* infection showing uninucleate forms (arrows) in the haemolymph. Scale bar = 10 µm. (**f**) Acute co-infection of the crab haemolymph; *Hematodinium* and multiple yeast like fungi (arrows). Haemocytes (Ha). Scale bar = 10 µm. Figure and legend from Davies et al. (2022).

Table 1. The main disease and disease-causing agents of shore crabs (*Carcinus maenas*)

Disease Causing Agent (and Diseases)	**Clinical signs of infection**	**References**
Viral conditions CmBV (*Carcinus maenas* bacilliform virus), CmPBV1 (*C. maenas* portunibunyavirus 1), HLV (Herpes-like virus), B virus, RVCM (Rod shaped virus of *C. maenas*), Y-organ virus, CHV (Crab haemocytopenic virus), parvovirus, iridovirus.	Infection of cytoplasm of hepatopancreas, gills, mid-gut, haemocytes. White haemolymph, induced haemocytopoenia.	(Bateman & Stentiford, 2017; Bojko et al., 2019; Bonami & Lightner, 2017; King et al., 2012)
Bacterial conditions Milky disease, *Vibrio* spp., Rickettsia-like organisms, shell disease syndrome.	Milky haemolymph, reduced haemocyte numbers, melanisation of hepatopancreas, rickettsia-like bacteria multiply in fixed phagocytes, darkened lesions on shell surface in shell disease.	(Bojko et al., 2018; Davies et al., 2019; Eddy et al., 2007; Rowley & Coates, 2023; Spindler-Barth, 1976; Wang, 2011)
Fungi (Mycosis)	Cloudy, viscous haemolymph, fungal elements in haemolymph, gills , intratubular space of the hepatopancreas, and free in connective tissue (Figure 4f)	(Davies et al., 2020b)
Microsporidians *Parahepatospora carcini, Agmasoma* sp., *Abelspora portucalensis*	Infection in the cytoplasm of epithelial cells of the hepatopancreas	(Azevedo, 1987; Bojko et al., 2017, 2023; Torchin, 2020; Stentiford & Dunn, 2014)
Haplosporidians *Haplosporidian cranc, H. carcini, H. littoralis*	Milky haemolymph, microcells in haemolymph (Figure 4e)	(Stentiford et al. 2004; Stentiford et al. 2013; Davies et al. 2020a)
Hematodiniosis (bitter crab disease) *Hematodinium* spp., *H. perezi*	Milky haemolymph, lethargy, parasites present in all tissues leading to eventual death (Figure 4a-c)	(Chatton & Poisson 1930; Coates et al., 2023; Davies et al., 2019; Hamilton et al., 2007; Rowley et al., 2015; Small et al., 2012; Stentiford & Shields, 2005)
Digenean trematodes *Maritrema portucalensis, M. subdolum, Microphallus claviformis, M. primas, M. similis*	Encysted metacercariae in hepatopancreas, gill, and muscle (Table 2; Figure 4d)	(Castilho & Barandela, 1990; Pina et al., 2011a, 2011b; Saville & Irwin, 1991, 2005; Stunkard, 1957; Zetlmeisl et al., 2011)
Turbellarians *Fecampia erythrocephala*	Development of worm larva in smaller crabs (CW <11mm)	(Kuris et al. 2002)
Parasitic barnacles *Sacculina carcini*	Internal root-like nodules, visible externa of abdomen	(Goddard et al. 2005; Powell and Rowley 2008; Larsen et al. 2013; Mouritsen et al. 2018; Rowley et al. 2020)
Acanthocephalans *Profilicollis botulus*	Formation of a cystacanth (parasite larval stage) within the crab's haemocoel.	(Thompson 1985a; Thompson 1985b)

1.2 The parasite

1.2.1 Life history and distribution of digenean trematodes

Digenean trematodes, classified within the class Trematoda of the phylum Platyhelminthes, are a diverse group of parasitic flatworms with complex life cycles that encompass a variety of vertebrate and invertebrate hosts (Olson et al. 2003; Arabuli et al. 2024). Like all parasites, these organisms contribute to the regulation of host population dynamics, thereby maintaining ecological balance within ecosystems (Dobson and Hudson 1986; Lafferty 2014). In marine ecosystems, digenean trematodes exhibit distinct morphological stages adapted to specific host requirements (Galaktionov & Dobrovolskij 2013; Galaktionov et al. 1997; Stunkard 1957). The distribution of these parasites is extensive, reflecting the native and non-native geographical ranges of their intermediate and definitive hosts (Blakeslee et al. 2015; Bojko et al. 2021; Cameron 1964; Pina et al. 2011a,b; Table 2). Moreover, factors such as salinity, temperature, and ocean currents influence the survival and dispersal of their free-living larval stages, altering host-parasite dynamics (Koprivnikar and Poulin 2009a; Galaktionov and Dobrovolskij 2013).

Table 2. Digenean trematodes of shore crabs (*Carcinus maenas*)

Genus, species	**Site(s)**	**Location of metacercaria in crab tissues**	**First intermediate host(s)**	**Final (definitive) host(s)**	**References**
Maritrema portucalensis	Aviero estuary, Portugal	Gill lamellae	*Peringia* (*Hydrobia*) *ulvae* (mudsnails)	Charadriifrom bird spp. (gulls, waders, shorebirds)	(Pina et al., 2011b)
M. subdolum	Various, including Wadden Sea	Gill lamellae	*P. ulvae*	Wading birds	(Thieltges et al., 2008; Zetlmeisl et al., 2011)
Microphallus claviformis	Wadden Sea	Interstitial tissue of hepatopancreas	*P. ulvae*		(Thieltges et al., 2008; Zetlmeisl et al., 2011)
M. primas	U.K. (various regions including South Wales, Clyde, Forth, Southampton, Northern Ireland). Aviero estuary, Portugal.	Interstitial tissue of hepatopancreas and gills	*P. ulvae*	Herring gulls (*Larus argentatus, Larus cachinnan*), Wading birds (*Haematopus ostralegus, Somateria mollissima*)	(Castilho & Barandela, 1990; Pina et al., 2011a; Saville & Irwin, 1991, 2005; Stentiford & Feist, 2005; Tkach et al., 2003)
M. similis	North America (Atlantic coast), U.K.,	Interstitial tissue of hepatopancreas and gills	Littorinid snails (including *Littorina saxatalis, L. littorea, L. obtuse*)	Herring gulls (*Larus argentatus*), Common tern (*Sterna hirundo*).	(Blakeslee et al., 2009, 2015; Bojko et al. 2018; Elner & Raffaelli, 1980; Galaktionov et al., 2012; Stentiford & Feist, 2005; Stunkard, 1957)
Gynaecotyla adunca (= ***Cercaria sevillana***, *G. longiintestinata*)	Aviero estuary, Portugal	Antennal gland, interstitial tissue of hepatopancreas	*Tritia reticulata* (*Nassarius reticulatus*), *Ilyanassa obsoleta*	Shore birds & fish	(Barnard, 2018; Hunter & Vernberg, 1953; Pina et al., 2007)

As shown in Table 2, both species from the genus *Maritrema* and *Microphallus* have been found in shore crabs together with isolated reports from the microphallid, *Gynaecotyla adunca* in crabs from the Averio estuary in Portugal (Pina et al., 2007). The most commonly reported microphallid, however, is *M. similis* that is found in both native and non-native shore crabs in the U.K. and North America. While the identification of these encysted microphallids is mainly using modern PCR-based taxonomic approaches of sequences in the ITS-1 region of rDNA, in some cases morphology alone is used (e.g. Stentiford and Feist 2005) and this can lead to errors in their identification. Additionally, as digenean identification has been subjective in the past this can result in misnaming as in the case of *G. adunca* with its junior synonyms of *Cercaria sevillana* (Pina et al., 2007; Russell-Pinto & Bartoli, 2002) and *G. longiintestinata* (Pina et al., 2007).

1.2.2 Life cycle of digenean trematodes

Typically, the life cycle of digenean trematodes involves three hosts, each crucial for progressing through their developmental stages (Figures 5 & 6). For these species, development begins in the gonadal tissue of gastropods, where prolific asexual reproduction occurs, leading to the continuous release of cercariae into the external environment (McCarthy et al. 2002; Blakeslee et al. 2015; Byers et al. 2016). These free-living larval stages actively seek out an intermediate host, such as fish, bivalves, and crustaceans (McCarthy et al. 2002; Saville and Irwin 2005). Notably, cercariae can circumvent the physical defences of crustaceans by exploiting respiratory currents to anchor onto haemolymph channels within the gills (Saville and Irwin 2005). Subsequently, cercariae migrate to the hepatopancreas and encyst as metacercariae, a morphological stage where the nutrient dense organ provides the optimal locale for dormancy and growth (Galaktionov et al. 1997; Huang et al. 2020). Observations of metacercarial cysts have also been reported in alternative tissues, including muscle and gill lamellae, which may be indicative of accidental tissue selection (Pina et al., 2011a; Saville & Irwin 2005; Stentiford and Feist 2005). The final transition to the definitive host, typically birds, occurs upon consumption of the parasitised intermediate host (Blakeslee et al. 2015).
This initiates the excystation of the metacercariae within the bird's digestive tract, where they quickly mature and begin sexual reproduction (Galaktionov et al. 2012). Trematode eggs are subsequently re-introduced into the environment through the excretion of the host's faeces (Saville and Irwin 1991; McCarthy et al. 2002; Galaktionov and Dobrovolskij 2013).

In contrast, the two-host life cycle of some digenean trematodes simplifies transmission by eliminating a host, thereby increasing the frequency of life cycle completion (Poulin and Cribb 2002). Host reduction can be achieved in multiple ways (Poulin and Cribb 2002). For example, *Microphallus piriformes* also uses the first intermediate host, *Littorina saxatilis*, as the secondary intermediate host

(Granovitch et al. 2004). Cercariae remain within the host gonadal tissue, where they encyst as metacercariae, before being ingested by the definitive host (Poulin and Cribb 2002). It is hypothesised that this strategic simplification in the life cycle may confer an evolutionary advantage by reducing the vulnerability to host viability fluctuation (Poulin and Cribb 2002; Granovitch et al. 2004).

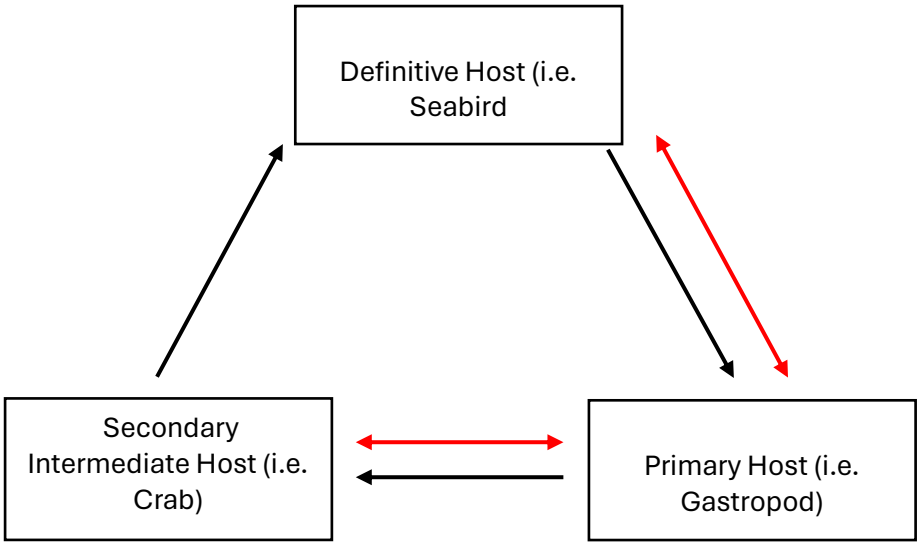

Figure 5. Life cycle of two-host (red arrows) and three-host (black arrows) digenean trematodes. Redrawn and adapted from Blakeslee et al. (2015).

Figure 6. Microphallus similis three host life cycle. (A) It begins with two species of littorinid snails (*L. saxatilis* and *L. obtusata*), where asexual reproduction occurs, producing cercariae. (B) These cercariae are released into the water column, encysting as metacercariae in a second intermediate host, primarily the green crab (*Carcinus maenas*). (C) Sexual reproduction occurs when the crab is ingested by a definitive host, typically a Larus gull species. (D) The trematode's eggs are then excreted into the marine environment with the bird's faeces, where they are ingested by grazing snails, continuing the cycle. Figure and legend from Blakeslee et al. (2015).

1.3 The responses

1.3.1 Physiological responses

Crustacean hosts, serving as intermediary carriers in the life cycle of digenean trematodes, undergo some variable physiological effects, ranging from minor to extreme (Chubb et al. 2010; Blakeslee et al. 2015). These disruptions are primarily characterised by impairment in organ function, reduced tissue integrity, and general health decline (Díaz et al. 2004; Chubb et al. 2010). For example, initial disruption of organ function is due to the penetration of cercariae (Saville and Irwin 2005). Respiratory current reversal is a feature of crab respiration (Arudpragasam and Naylor 1964). However, reports have demonstrated that *C. maenas* in the presence of *M. primas* cercariae, elicit an increase in current reversal frequency, perhaps indicating a disruption to gill function (Saville and Irwin 2005). The hepatopancreas of crustaceans is commonly the site of metacercariae and it is thought that they obtain nutrients from this nutrient-rich tissue (Sparks and Hibbits 1981; Castilho and Barandela 1990; Robaldo et al. 1999; Galaktionov and Dobrovolskij 2013; Blakeslee et al. 2015). In granulated crabs (*Chasmagnathus granulata*) the increased pressure exerted by cysts in the hepatopancreas has also been shown to result in the necrosis of the tubules (Robaldo et al. 1999).

1.3.2 Behavioural responses

Some parasites are capable of modifying host behaviours to coincide with the success of their own reproductive cycles through a process termed parasite-increased trophic transmission (PITT) (Lafferty 1999). However, for many parasites changes in host behaviour is not an active process, rather an incidental pathological effect caused by the parasite's physical presence (Leung and Poulin 2006). For instance, reports have documented the presence of trematode metacercariae in the cerebral regions of crustaceans, which may provide an explanation for atypical host behaviour (Sparks and Hibbits 1981; Hansen and Poulin 2005; Blakeslee et al. 2015). In freshwater isopods (*Austridotea annectens*), increased swimming activity and diminished evasion responses in the presence of predators were observed in trematode-encysted individuals (Hansen and Poulin 2005). Similarly, grass shrimp (*Palaemonetes pugio*) do not exhibit their typical behaviour of remaining motionless to avoid predator detection when encysted with *M. turgidus* (Gonzalez 2016). Conversely, shore crabs appear to have more nuanced behavioural responses following the acquisition of metacercariae by their tissues (Blakesee et al. 2015). Behavioural observations of the Dungeness crab (*Cancer magister*) encysted with the metacercariae of an unknown trematode species, revealed minimal response (Sparks and Hibbits 1981). However, it was inferred that metacercariae may potentially impede nerve transmission resulting in the lethargic behaviours observed in the crab host (Sparks and

Hibbits 1981). Heavily infected shore crabs with numerous *M. similis* metacercariae in their thoracic ganglia have also displayed an increase in the duration of mussel handling with infection intensity, suggesting an impairment in feeding activity (Blakeslee et al. 2015). These same crabs were also recorded as having a righting response 1.7 times slower than the average righting time for uninfected crabs (Blakeslee et al. 2015). Overall, however, parasitised shore crabs only appear to exhibit minor changes in behaviour suggesting that they are no more susceptible to predation by birds than uninfected individuals in the same population.

1.3.3 Host immune responses

Crustaceans deploy specialised immune responses against parasitic invasions, primarily through encapsulation (Rowley, 2024). This response is triggered when pathogen-associated molecular patterns are recognised by host proteins, resulting in the recruitment and activation of haemocytes to the affected tissue (Smith and Chisholm 1992; Vogt 2012). This has been demonstrated in shore crabs, where a decrease in circulating haemocyte density in the haemolymph has been reported 72 hr following exposure to *M. similis* cercariae (Blakeslee et al. 2015). In some cases, haemocytes infiltrate into encysted tissue surrounding metacercariae to elicit an acute encapsulation response (Martorelli & Schuldt 1990; Pina et al. 2011b). This often leads to the melanisation of the surrounding hepatopancreas foci (Stentiford and Feist 2005). However, the effectiveness of the encapsulation response is dependent on multiple factors: haemocyte recruitment to the affected tissue; tissue size; and the specific tissue characteristics (Martorelli and Schuldt 1990). For example, ineffective haemocyte recruitment has been observed in shore crab gills encysted with *M. primas*, characterised by the stasis of haemocytes in the gill lamellae (Stentiford and Feist 2005). Histological studies of encysted metacercariae in shore crabs show little evidence of any substantial encapsulation host response (Davies et al. 2020a: Figure 4d) perhaps suggesting a lack of recognition of these parasites by the host's immune system or some form of immune suppression (Rowley 2024).

1.4 The aims

The overall aim of this thesis is to investigate the presence of digenean parasites in *C. maenas* in two intertidal locations (Mumbles Head and Oxwich Bay) in South Wales, UK. These sites have been used in several studies of disease occurrence in both shore crabs and edible crabs (*Cancer pagurus*) over the last two decades (Davies et al., 2019; Davies et al., 2020a,b; Rowley et al., 2015, 2020; Vogan et al., 1999; Vogan & Rowley, 2002) and therefore the current study aims to add to this extensive disease dataset. Also, as an invasive species, it is crucial to quantify the prevalence of disease with the population, thereby informing future management strategies and conservation efforts in affected ecosystems.

The specific objectives of this thesis are as follows:

1. To identify the species of digenean trematodes present in *C. maenas* at Oxwich Bay and Mumbles Head in Swansea, UK.
2. To determine if shore crabs are hosts to more than one species of digenean parasite
3. To determine if parasite presence, and load correlates with biometric data taken from each crab (e.g. sex, moult stage, presence of fouling epibionts, limb loss, haemolymph colour).
4. To identify whether metacercarial cysts of the same species vary in size (and hence nutritional status?) across both locations.

Chapter Two

Materials and methods

2.1 Study area

The study was conducted at two coastal sites around Swansea, Wales: Oxwich Bay (51°33'11.4"N 4°09'03.7"W) and Mumbles Head (51° 34' 8.36"N, 3° 58' 35.93"W). Oxwich Bay, situated on the south shore of the Gower Peninsula primarily consists of sandy sediment, with subtidal rocky reefs to the southwest (collection site), and isolated saltmarshes and estuaries to the north (CEFAS, 2013). Mumbles head, situated on a rocky shore, is located to the south of Swansea Bay. Positioned within the Bristol Channel, both shores feature substantial tidal ranges (8.5m spring tides; 4.1m neap tides) (Collins et al. 1979).

2.2 Sample collection

Crab collection was conducted at both locations three times over a six-month period (April, June, and August 2024). Approximately 30 shore crabs were randomly sampled in the intertidal zone during each collection event. At Oxwich Bay, samples were collected exclusively using manual collection by boulder turning. In contrast, at Mumbles, an additional collection method was employed: baited crab pots, which were deployed and immersed 24 hr prior to collection. Surveyed crabs were transported back in seawater to an aquarium where they remained for > 48 hr. During this time crabs were fed with mussels.

2.3 Laboratory regime

All *C. maenas* were processed > 48 hr post collection and placed on ice for a minimum of 20 min prior to examination. Biometric data were recorded for each crab. These included sex (male or female); moult stage [inter-moult (hard) or post-moult (soft)]; fouling (visible epibionts on the crab surface); external presence of potential disease (*Sacculina*, pigment loss, shell disease); carapace width (CW; mm); ventral carapace appearance (green, yellow, orange/red); limb loss or additional damage (e.g., exoskeletal fractures) and weight (g). Approximately 300 µl of haemolymph were extracted from each crab using a 23-guage hypodermic needle fitted to a 1 ml syringe. Haemolymph appearance was deemed either ‘normal’(clear to slightly cloudy) or milky in appearance as an initial indication of potential infection. A small drop of haemolymph was then placed on to a microscope slide for screening using phase contrast optics of a BX41 microscope (Olympus, Tokyo, Japan). Haemolymph preparations were assessed for the presence of *Hematodinium*, haplosporidians, and

fungi based on their morphology. The remaining extracted haemolymph was centrifuged for at 6000 x g for 4 min and stored at – 18℃ for potential further analysis (not included in this thesis).

2.3.1 Initial protocol (February 2024 only)

Initial experiments were carried out on a small number of crabs (n = 13; not included in full dataset) collected in February to validate the methods to be used in the main collections in April – August. In these, crabs were euthanised via injection of 0.5-1 ml of 1M KCl (or until involuntary motor functions were absent). Scissors were used to separate the main body from the ventral surface and limbs. Approximately 0.05 – 0.25g of tissue was extracted from four separate regions of the hepatopancreas to find encysted metacercariae and placed on separate slides. Each slide was weighed to determine the hepatopancreas wet weight. Tissue was then squashed using a 20x50 mm coverslip and examined under a GX dissecting microscope. The presence and quantity of metacercarial cysts in each sample was recorded. Identified metacercaria were extracted from the squashed hepatopancreas tissue, using fine forceps, and stored in a 1.5 ml Eppendorf tube at – 18℃ for later nucleic acid extraction.

2.3.2 Finalised protocol (April – August 2024)

Following this initial trial, a few changes were made in the processing regime for all subsequent data collection between April and August. In the revised protocol, crabs were sacrificed via placing at – 18℃ for 30-45 min (or until involuntary motor function was absent). This change was made for two reasons; (1) to eliminate the potential osmotic effect of KCl, which could alter metacercarial cyst size, and (2) because euthanasia via cooling to – 18℃ is a preferred method in some literature (Naczk et al. 2004; Penney et al. 2016; Coyle 2017). Alcohol-sterilised scissors were used to separate the carapace body from the ventral surface at the moult line. The whole hepatopancreas was removed and placed into a pre-weighed 50 ml Falcon tube. The weight of the tube containing hepatopancreas was then recorded and stored at – 18℃ for later processing. Subsequently, hepatopancreas samples were removed and left to thaw at RT for 30 min. Each tissue sample was then dissociated in 4-5 ml of 3% NaCl and vacuum filtered through a sterile 150 µm pore size low density polyethylene cell strainer (pluriStrainer, Leipzig, Germany). Metacercarial cyst counts were performed on the filter using a dissecting microscope. Metacercariae from positive samples removed from the filter, placed into a 1.5 ml Eppendorf tube, and stored at – 18℃ for later DNA extraction. This revised protocol was adopted to improve the accuracy of cyst counts and to ensure all metacercarial cyst species were correctly identified, thereby providing an accurate survey of

the presence of digenean trematodes in crabs across the two locations. A small number of cysts (n = 134) were documented using the x4 and x10 objectives of an Olympus BX41 microscope equipped with a digital camera. Cysts sizes were calculated using imageJ (Schindelin et al. 2012) by measuring height and width of the inner most cyst wall.

2.4 DNA extraction and quantification

Small groups of metacercariae were removed from the dissociated hepatopancreas viewed under a low power dissecting microscope. DNA was extracted from thawed samples using a Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany) with adapted manufacturer's instructions by extending proteinase K digestion to overnight. Extracted DNA was quantified using Qubit® dsDNA Broad Sensitivity Assay Kit and Qubit® Fluorometer (ThermoFisher Scientific, Altrincham, UK).

2.5 PCR and sequencing conditions

All PCR reactions were carried out in 25 µl total reaction volumes containing 12.5 µl of 2 x BioMix (New England Biolabs Inc., Ipswitch, USA), 0.5 µl of each primer at concentration of 10 µM (Eurofins, Ebersberg, Germany), 10.5 µl of nuclease-free water (Invitrogen™, Leicestershire, UK), 1 µl of genomic DNA (*ca*. 50 – 200 ng/µl) and performed on a T100 PCR thermal cycler (BioRad Laboratories Inc., Watford, UK). Universal digenean primers derived from Tkach et al. (2003) and Galaktionov et al. (2012) were employed. These were forward primer LSU-5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3') and reverse primer LSU – 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3'). The cycling conditions were as follows: 3 min denaturation hold at 94℃; 35 cycles of 30 sec at 94℃, 30 sec at 60℃, 1 min at 72℃; and a final phase at 72℃ for 5 min.

5 µl of post PCR product was mixed with 1 µl of 6x DNA loading dye (ThermoFisher Scientific, Altrincham, UK) and loaded on to a 2% agarose/TAE gel stained with GreenSafe premium nucleic acid stain (NZYTech, Lisboa, Portugal). Gels were run for 45-60 min at 60 volts with a 1 kb Plus DNA Ladder (New England Biolabs, Hitchin, UK). Gel imaging was completed using a Molecular Imager® Gel Doc™ XR System (BioRad Laboratories Inc., Watford, UK). Positive samples were purified using ExoSAP-IT™ Express Fast High-Throughput PCR product cleanup (ThermoFisher Scientific, Altrincham, UK) for target sequencing (5:2; 4 min at 37°C, 1 min at 80°C). Amplicons were sent for DNA Sanger sequencing using both forward and reverse primers,

synthesised by Eurofins (Ebersberg, Germany). Sequence alignments were performed using BioEdit (Hall et al. 1999) and subjected to additional analysis using the bioinformatic tool for similarity search BLAST (Camacho et al. 2009).

2.6 Phylogenetic analysis

Sequences were edited to remove primers and consensus sequences were constructed using the CAP contig assembly extension in BioEdit sequence alignment editor (Hall et al. 1999). Reference sequences of the respective region from *M. similis* and *M. primas* recovered a broad range of *Microphallus* sp. hosts were sourced from Genbank (*L. schistisagus, P. brazieri, L. sitkana, L. saxatilis, S. mollissima, L. natica, P. ulvae, Juga* sp.*, I. manipurense*) (See appendix, Table A4). Sequence alignments were performed using BioEdit (https://bioedit.software.informer.com/) and subjected to additional analysis using the bioinformatic tool for similarity search BLAST (Camacho et al. 2009). Multiple sequence alignments were performed in CLUSTAL X v.2 (Larkin et al. 2007). Evolutionary analyses and reconstructions were carried out in MEGA X (Kumar et al. 2018) using the maximum likelihood routine based on the Tamura-Nei model. This model was selected due to its proven effectiveness in previous research (Davies et al 2020a, Davies et al 2019). A consensus tree with the highest log likelihood value from 1000 bootstrap re-samplings was annotated using ITOL software (Letunic and Bork 2019). All sequences have been deposited in the GenBank database under the accession numbers PQ314574 – PQ314610 (See appendix, Table A3)

2.7 Statistical analysis

Binomial logistic regression models with Logit link functions (following Bernoulli distributions) were used (MASS library) to examine the impact of specific predictors on the likelihood of detecting digenean trematodes in sampled crab populations. Initially, all potential predictor variables were included in what are termed full models. Non-significant predictors were then sequentially eliminated using the drop1 function to develop reduced models with greater predictive accuracy. The drop1 function tests each reduced model, lacking the least significant predictor, against the original full model. If a reduced model shows a statistically significant difference (using a Chi-square test for binomial responses to evaluate differences in residual sum of squares), the excluded predictor is permanently omitted. This stepwise refinement continues until a final, optimized model is established. The full models included the input variables: month (April, June, August), carapace width (CW)(continuous number), sex (male or female), colour (green, yellow, red), pigment loss (0 or 1),

haemolymph opacity (clear or milky, 0 or 1), fouling (presence of epibionts, 0 or 1). Initial model also included location (Mumbles or Oxwich) before being separate for further analysis.

To examine the impact of specific predictors on metacercarial cyst load within the sampled crab population, a Generalized Linear Model (GLM) with a Negative Binomial function was utilized (MASS library). This model was selected due normality tests indicating that the data presented with severe overdispersion (variance > mean). Prior to analysis, the dataset was refined using the subset function to exclude crabs with zero cysts. The input variables included in the model were as follows: location (Mumbles, Oxwich), month (April, June, August), carapace width (CW) (continuous number), sex (male or female), haemolymph opacity (clear or milky, 0 or 1), fouling (presence of epibionts, 0 or 1), and pigment loss (0 or 1).

A Mann-Whitney test was conducted to determine if there was a statistically significant difference in the average cyst size (calculated as height x width) between two sample locations, Mumbles and Oxwich. Normality of the dataset was assessed using a Shapiro-Wilk test. This analysis was restricted to metacercariae with a spherical morphology only. The measurements of non-spherical metacercarial cysts were reported as height and width only. Graphics and statistical analysis were performed using GraphPad Prism v10.0.0 for Mac OS X and RStudio (v. 2024.04.2+764) on R (v.3.6.0).

Chapter Three

Results

3.1 Population observations

3.1.1 General population observations and cyst morphology

In total, 163 crabs were sampled between April-August, 75 from Mumbles and 88 from Oxwich. Of these crabs, examination of hepatopancreas samples under a binocular microscope revealed that 75.5% over the two sites were infected with trematode metacercariae. Cysts examined from the February (initial trial) and April (main trial) were spherical and all of similar size and morphology (Figure 7a, c). Two morphologically distinct types of metacercariae were found starting from June onwards, with spherical forms as seen in February and April and new asymmetrical forms found in only 9.3% of infected crabs from Mumbles and 2.3% from Oxwich (Figure 7b, c). Notably, these distinct asymmetrical metacercariae were mostly observed in low intensity infections and always appeared as joint infections with the spherical form metacercariae. Finally, there was no evidence of any cellular host response, specifically involving melanisation of the cysts, in any of the metacercariae examined.

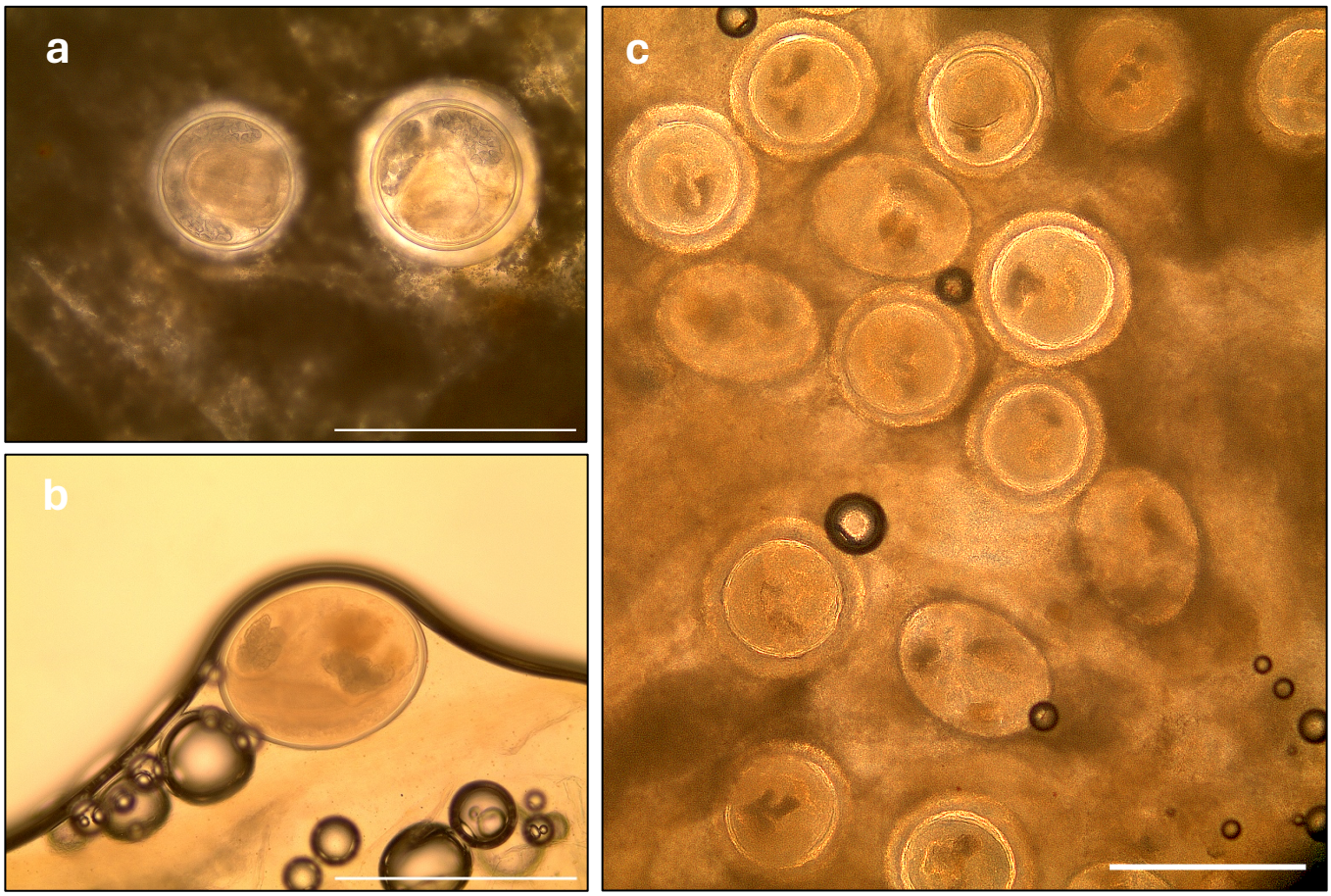

Figure 7. Variable morphology of digenean metacercariae in fresh hepatopancreas preparations. The majority of crabs contained rounded metacercariae (**R**) as seen panels **a** and **c**. A small number of crabs had asymmetrical forms of a different size, morphology, and thinner outer layer than spherical forms as seen panels **b** and **c**. The crab in panel **c** had a high intensity infection with large numbers of clustered metacercariae. Scale bars = 500 µm. Figure 7a – darkfield and Figures 7b – c brightfield microscopy.

Haemolymph screening using phase contrast microscopy identified *Hematodinium* infections in 10.4% of crabs, with infection severity ranging from low (levels L1 and L2) to high (levels L3 and L4) (see Davies et al., 2019 for a description of the severity levels) (Figure 8b). Additionally, 1.2% of crabs were found to have a *Haplosporidian carcini* infections (Figure 8a), and 1.8% were diagnosed with an unidentified fungal infection. No crabs were found with externa of *S. carcini* and while some crabs were found with shell disease lesions, these were all superficial in nature (See Appendix for full biometric data, Table A1)

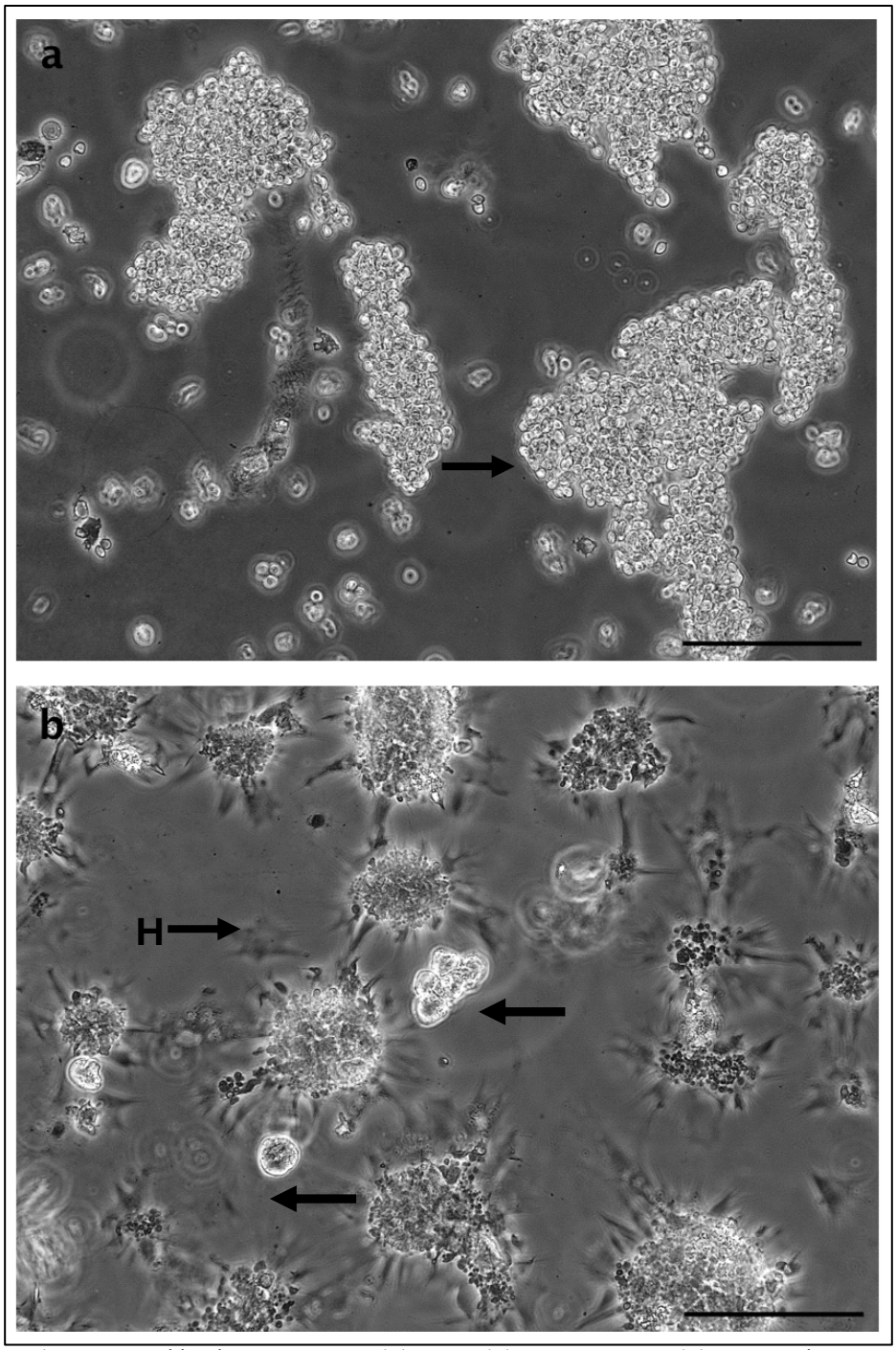

Figure 8. Identification of *Haplosporidium carcini* (**a**) and *Hematodinium* sp. (**b**) in fresh haemolymph preparations using phase contrast microscopy. (**a**) High-grade infection with uninucleate parasites (arrow) and a low number of haemocytes. (**b**) Highly refractile *Hematodinium* parasites (arrows), identified by their lack of attachment and spreading to slides (*cf.* haemocytes, H), with irregular sizes and shapes. Scale bars = 100 µm.

Model 1, a binomial logistic regression model, which combined data from both locations and used the presence of trematode parasites as the response variable, showed that size (CW) was the only factor associated with the presence of parasites (Table 3, Model 1). In terms of size (CW), larger crabs were significantly more likely to present with trematode infections compared to smaller crabs (mean ± SD: 41.1 ± 10.6 *vs* 32.8 ± 10.9 mm, respectively; Figure 9a). The prevalence of trematode infections in crabs sampled from Mumbles and Oxwich was 85.3% and 67.0%, respectively. The drop1 function revealed that despite an apparent lower infection presence at Oxwich, location was not an associated factor with trematode presence. Of the male crabs, 68.6% were parasitised whereas 83.1% of females presented with trematode metacercariae (Figure 10d). Although this may suggest a higher prevalence of infection in female crabs, the combined model showed that sex was not a significant factor associated with trematode presence (Table 3, Model 1).

3.1.2 Population observations by location

To further explore the possible relationship between external factors and the presence of trematode parasites, the data were separated and analysed between the two locations (i.e. Mumbles *vs.* Oxwich). In Mumbles, 85.3% of surveyed crabs presented with trematode metacercariae. Model 2, which used the presence of trematode infection at Mumbles as the response variable, indicated that no factors were associated with infection. In terms of size (CW), the average carapace width of infected crabs from Mumbles was 43.3 ± 10.3 mm (range 19 – 61 mm). Although the combined model (Model 1) identified crabs of a larger size (CW) as a factor associated with trematode presence, subsequent analysis using the drop1 function in Model 2 deemed that size was not statistically significant for inclusion in the final (reduced) model (Table 1, Models 1 – 2; Figure 9b). The drop1 function indicated a lower presence of infection during August, however, the significant threshold ($p = 0.05$) was not achieved (Table 1, Model 2; Figure 10b). Additionally, sex, fouling (presence of epibionts), pigment loss, haemolymph opacity, and limb loss did not have a significant effect (See appendix, Table A2)

Of the crabs surveyed from Oxwich, 67.0% presented with trematode metacercariae in the hepatopancreas. Using the presence of trematode infections at Oxwich as the response variable (Model 3), revealed that size (CW) and colouration (ventral surface) were significant factors associated with trematode presence (Table 1, Model 3). In terms of size, larger crabs were significantly more likely to be infected with trematode parasites compared to smaller crabs (mean ± SD: 38.7 ± 10.5 *vs.* 29.9 ± 8.0 mm, respectively, Figure 9c). Of the crabs surveyed from Oxwich, the colour (ventral surface) distribution of infected individuals were as follows: 42.4% green, 6.8% yellow, and 50.8% red. Notably, shore crabs that presented with a yellow carapace colouration were significantly less likely to host trematode parasites (Table 1, Model 3; Figure 10i).

Table 3. Binomial logistic regression models (reduced from full models) testing the effects of biometric and environmental predictor variables on the overall presence of trematode parasites in the population. Models separated by location: Model 1, total population; Model 2, Mumbles; Model 3, Oxwich.

Model	Predictor Variable	Estimate (slope)	SE	P-value
Model 1				
TremPres ~ Location + Sex	Location (Oxwich)	- 0.71784	0.42619	0.09212 .
+ Carapace Width	Sex (Male)	-0.55100	0.41173	0.18082
df = 159	Carapace Width	0.05918	0.01984	0.00286 **
AIC: 167.77				
Model 2				
TremPres ~ Month	Month (August)	-2.3506	-1.714	0.08653 .
+ Colour	Month (June)	-0.2323	-0.275	0.78343
+ Hemo.col	Colour (Red)	-2.1355	-1.596	0.11047
df = 69	Colour (Yellow)	15.3151	0.007	0.99426
AIC: 69.402	HemoCol (Milky)	0.8920	0.962	0.33594
Model 3				
TremPres ~ Carapace.Width	Carapace Width	0.14027	0.04098	0.00062***
+ Colour	Colour (Red)	0.76112	0.59952	0.20424
df = 84	Colour (Yellow)	-2.53315	1.16239	0.02931*
AIC: 94.678				

Statistically significant *$P \leq 0.05$, **$P \leq 0.01$,$P \leq 0.001$**

Abbreviation: SE, standard error

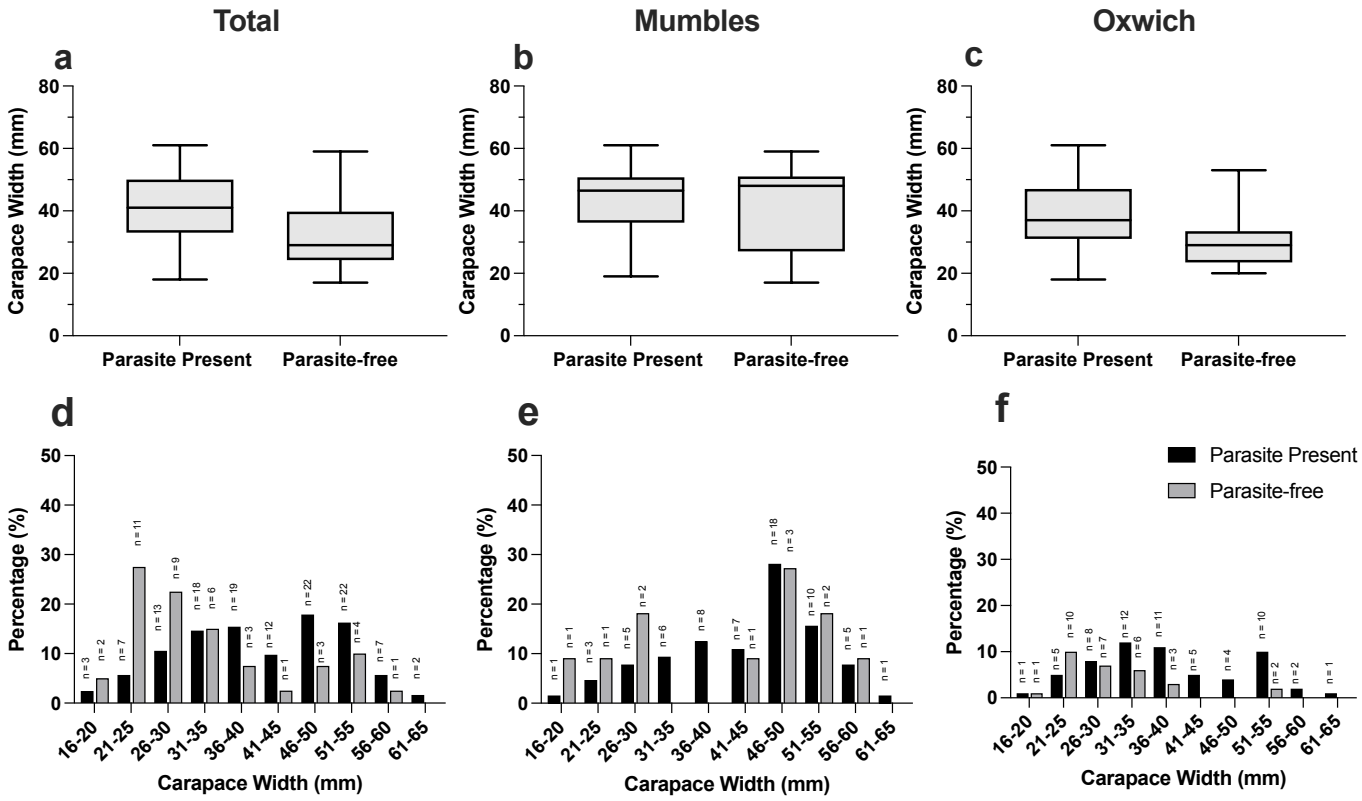

Figure 9. Carapace width (mm) of *C. maenas* parasitised with digenean trematodes ('parasite-present') and those 'parasite-free' per location: total population (**a**), Mumbles (**b**), and Oxwich (**c**). Also shown, size distribution in 5 mm size groups of the percentage of *C. maenas* infected ('parasite present') and those 'parasite-free' per location: total population (**d**), Mumbles (**e**), and Oxwich (**f**).

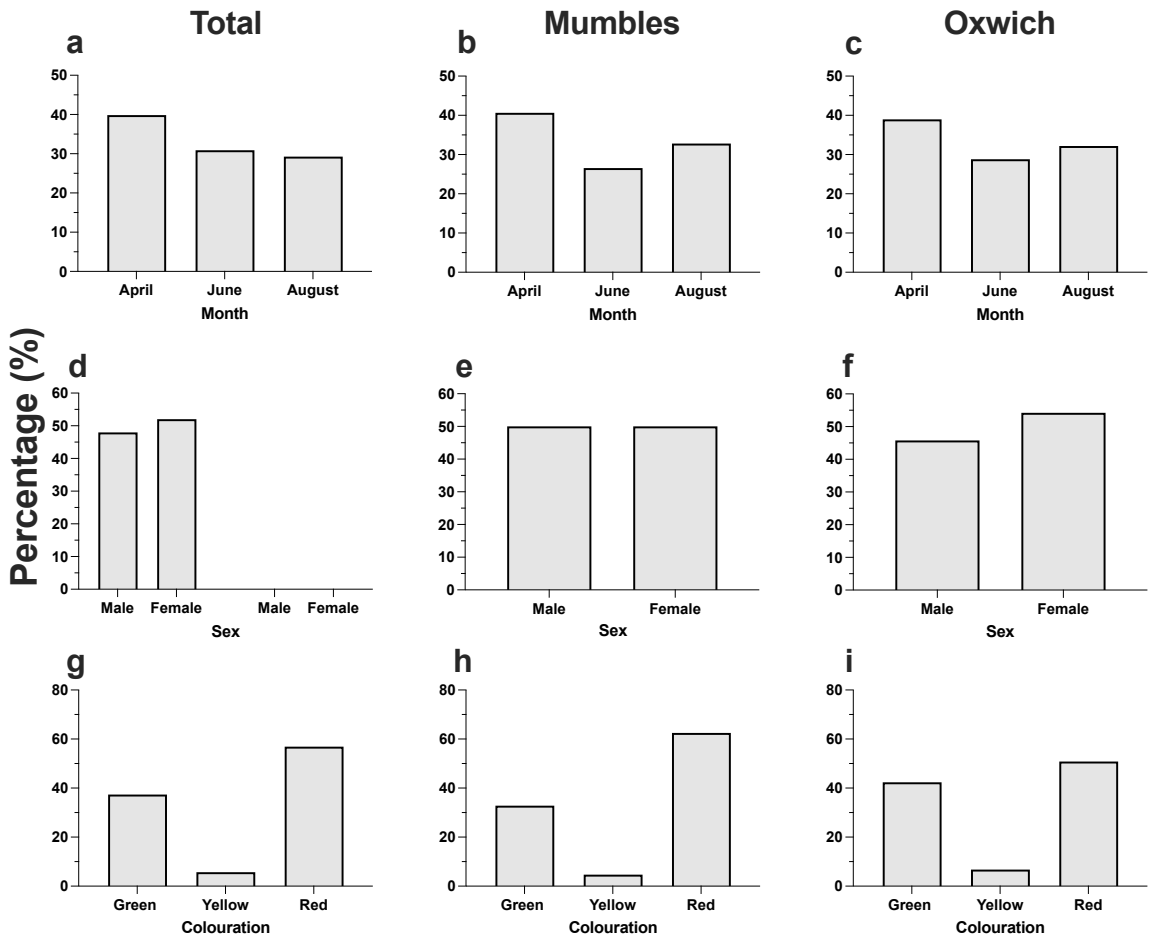

Figure 10. Percentage of digenean trematode infections in *Carcinus maenas* per location: total population, Mumbles and Oxwich, according to their predictor variables month (**a** – **c**), sex (**d** – **f**), and coloration (**g** - **i**).

3.2 Metacercariae Infection Intensity

Infection intensity, which was parasite count (load) per crab was also investigated. Model 4, which combined data from both locations and used parasite count (load) as the response variable, showed that location, carapace width (CW), and fouling as the only factors that had a significant effect on parasite load (Table 4, Model 4). In terms of size, the model showed that larger crabs were significantly more likely to harbour a greater number of parasites (Table 4, Model 4). Crabs sampled from Oxwich had a significantly lower parasite count compared to Mumbles (mean ± SD: 24.9 ± 39.9 vs 40.8 ± 50.7, respectively; Figure 12a). In terms of month, there was no significant difference in the number of parasites per crab across sampling months (April, June, August) (mean ± SD: 31.3 ± 46.6 vs 42.4 ± 49.2 vs 25.91 ± 42.5, respectively; Table 4, Model 4; Figure 12b). Model 4 showed no significant difference in the number of parasites per crab when comparing males and female (mean ± SD: 28.8 ± 48.6 vs 37.1 ± 44.2, respectively; Figure 12c). In terms of fouling, the model indicated that crabs with epibionts were significantly more likely to have a greater parasite load than those without epibionts (mean ± SD: 51.8 ± 57.4 vs 29.8 ±43.6, respectively, Table 4, Model 4; Figure 12d). Furthermore, haemolymph opacity, which can be an early indication of systematic infection, had no association with a change in parasite load (mean ± SD: 32.8 ± 46.6 vs 37.3 ± 46.0, clear and milky, respectively; Table 4, Model 4).

Table 4. Generalised linear model with a negative binomial function testing the effect of biometric and environmental predictor variables on digenean trematode metacercarial cyst load in the total population of sampled *C. maenas*.

Model	Predictor Variable	Estimate (slope)	SE	P-value
Model 4				
Parasite count ~ Location + Month + Sex + Carapace Width + HemoCol + Pigment Loss + Fouling	Location (Oxwich)	-0.79164	0.23512	0.00076 ***
	Month (August)	-0.46575	0.27428	0.08949 .
	Month (June)	-0.22969	0.26869	0.39263
	Sex (Male)	-0.44703	0.22956	0.05150 .
	Carapace Width	0.03817	0.01204	0.00153 **
	HemoCol (Milky)	-0.37159	0.46040	0.41960
df = 116	Pigment Loss	0.02777	0.27760	0.92031
AIC = 1082.5	Fouling	0.81158	0.34743	0.01949 *

Statistically significant *$P \leq 0.05$, **$P \leq 0.01$,$P \leq 0.001$**

Abbreviation: SE, standard error

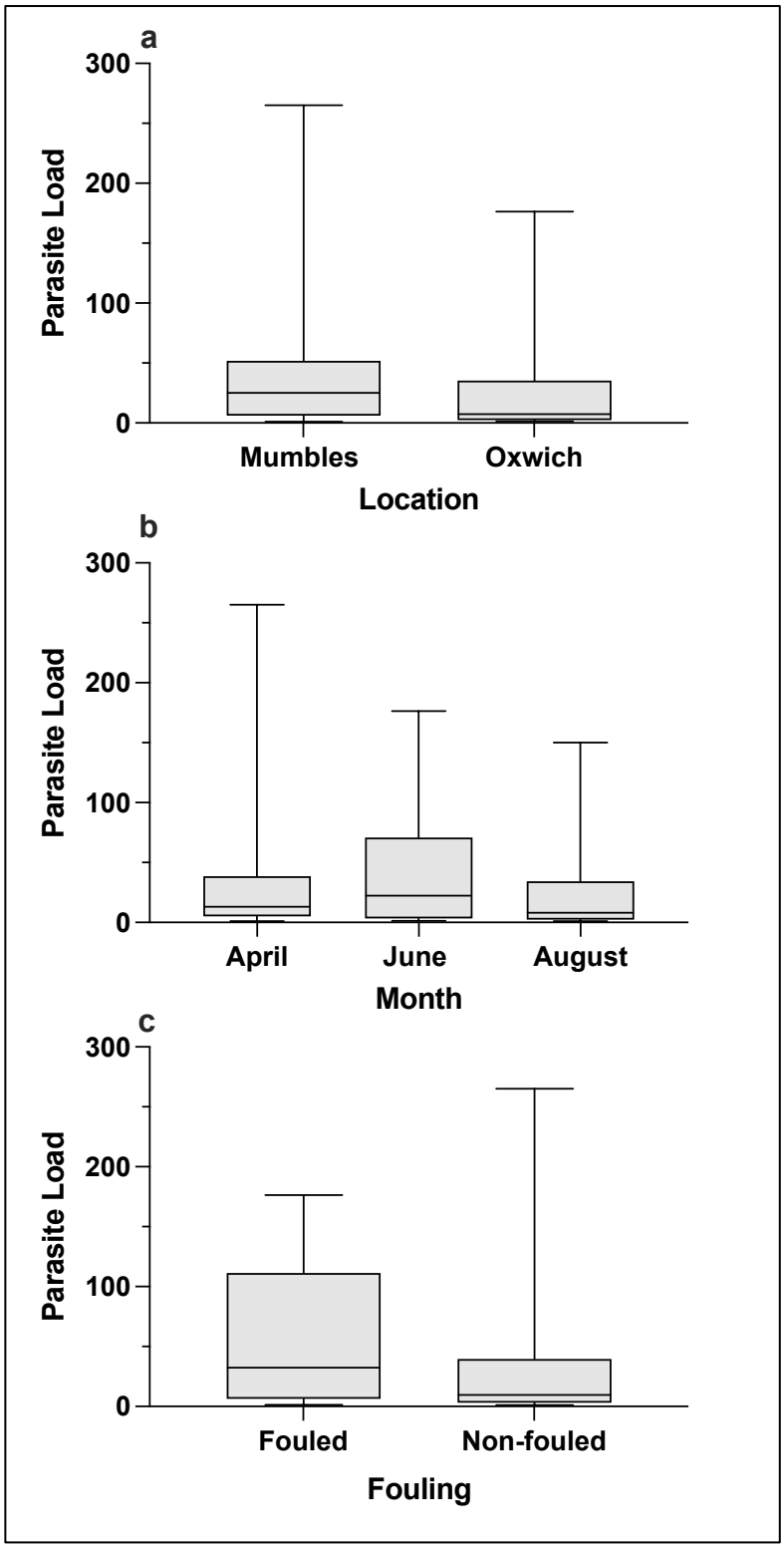

Figure 11. Digenean trematode metacercarial load in *C. maenas* according to significant predictor variables: location (**a**), month (**b**), and fouling (**c**). Boxplots display the median, interquartile range, and whiskers extend to the minimum and maximum observed values.

3.3 Metacercarial cyst sizes

A total of 134 digenean trematode encysted metacercariae were measured. Among these, 120 from Mumbles (n=87) and Oxwich (n=35) were identified as spherical metacercariae consistent with the morphology described for *Microphallus similis* (Stunkard, 1957; Figure 7a). Normality of the dataset was assessed using a Shapiro–Wilk test, which indicated that the data were not normally distributed ($P < 0.05$), and therefore a Mann–Whitney U test was conducted to determine whether there was a statistically significant difference in the size of M. similis metacercariae between the two sample locations, Mumbles and Oxwich. This test indicated that there was a significant difference in cyst sizes between location ($U = 1126$, $n_1 = 87$, $n_2 = 35$, $P = 0.0244$), with significantly larger metacercariae at Mumbles when compared to Oxwich (mean ± SD: 291.8 ± 39.7 *vs.* 271.9 ± 22.9 µm, respectively). The remaining measured metacercariae were identified by their oval (asymmetrical) morphology synonymous with descriptions of *M. primas* (Pina et al., 2011a; Figure 7b). Due to an insufficient number of metacercariae samples, no statistical analysis was performed on cysts with oval-shaped morphology. General population measurements (n = 14) for combined metacercarial sizes showed mean dimensions (height *vs.* width) of 406.7 ± 16.2 by 313.3 ± 16.8 µm, respectively.

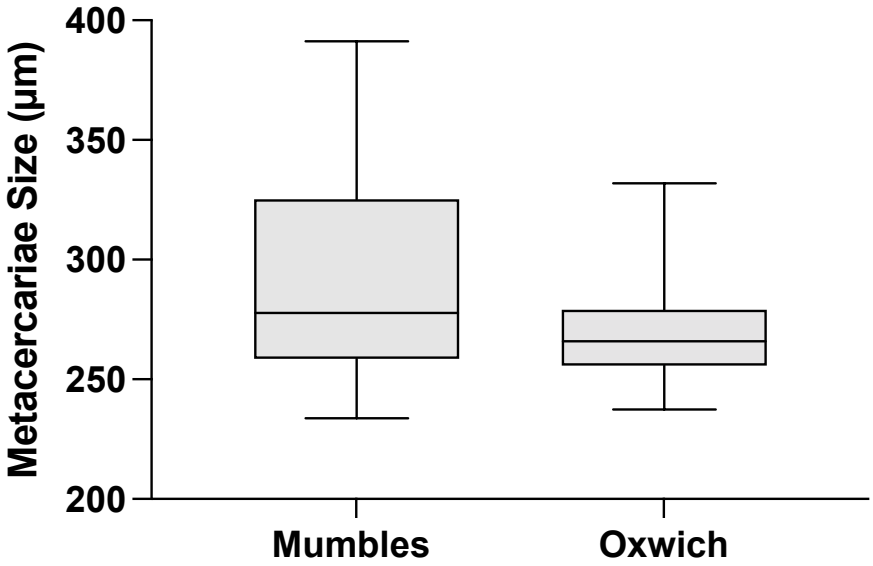

Figure 12. Size comparison (µm) of spherical trematode metacercariae extracted from the hepatopancreas of *C. maenas* sampled from two locations: Mumbles and Oxwich Bay. Boxplots display the median, interquartile range, and whiskers extend to the minimum and maximum observed values.

3.4 Phylogenetic Analysis

Of the 123 trematode-infected crabs, a total of 26 samples from Mumbles (n= 15) and Oxwich (n = 11) were successfully re-amplified and sequenced using the LSU-F/LSU-1500R oligonucleotides. Following quality control, all of these sequences (of the partial 28S rRNA gene regions of digenean trematodes) were combined with 37 reference sequences for evolutionary analysis (Figure 13) and deposited in the Genbank database under the accession numbers PQ314574 – PQ314610 (See appendix, Table A3). Of these sequences, 95.7% shared considerable high similarity (>98.0% cover and identity) with *M. similis* from a slaty-backed gull (*L. schistisagus*) in the Sea of Okhotsk, Russia (GenBank: HM584136 - HM584138, See Appendix, Table A4). The remaining sequences derived from crabs harbouring the less common asymmetrical cysts also shared considerably high similarity (>94% cover and identity) with *M. primas* from the mudsnail (*P. ulvae*) (See appendix for sequence alignment, Figure A1). The derived phylogram distinctly separates *M. similis* and *M. primas* into individual clades. Notably, the majority of the *M. similis* sequences formed a robust clade. In contrast, three *M. primas* sequences from August were unified in a single clade, while the one sequence from June displayed phylogenetic separation, suggesting potential genetic variation within the species or even another species.

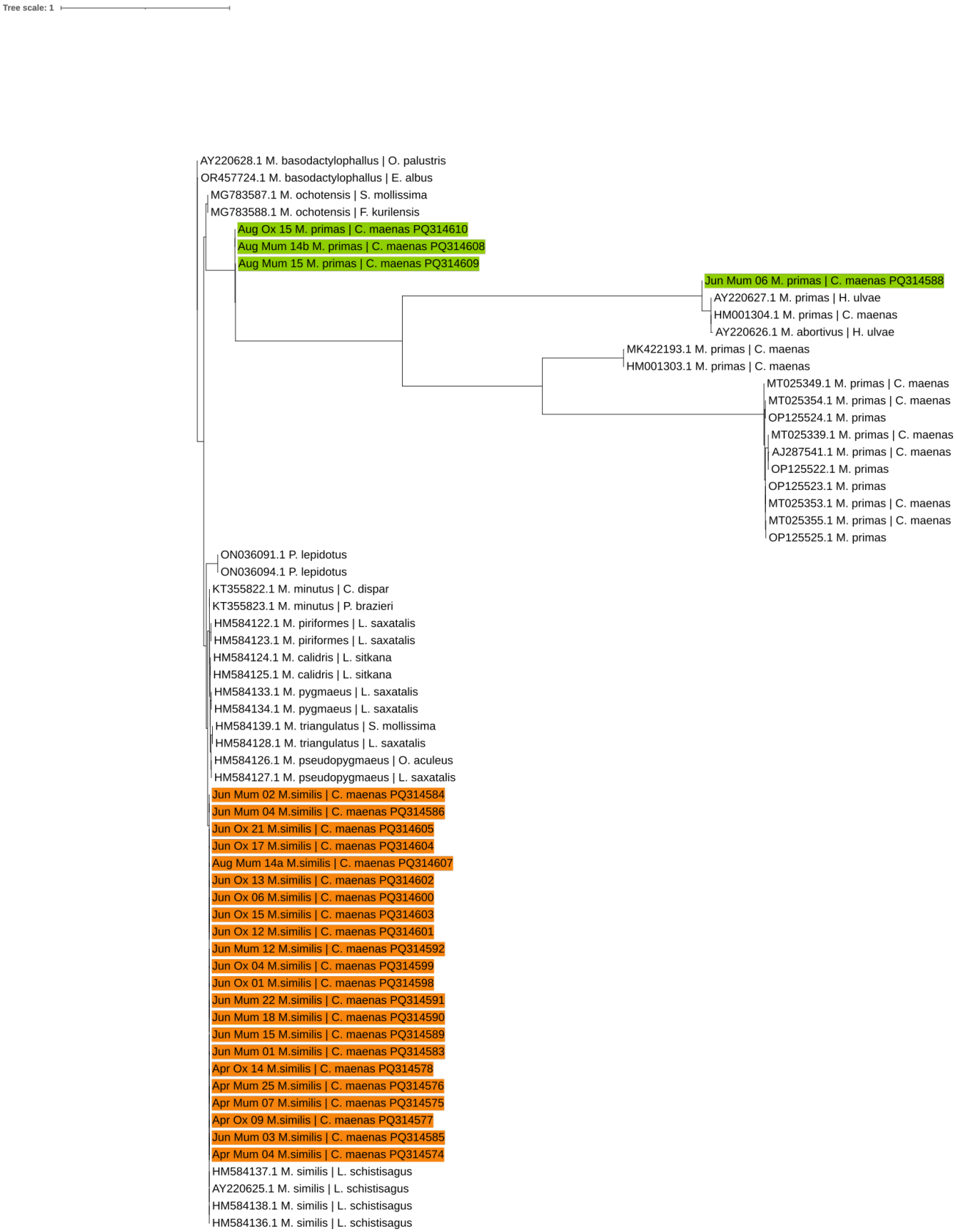

Figure 13. Consensus phylogram of the partial 28S rRNA gene regions from trematode parasitised shore crabs (*C. maenas*) (maximum likelihood estimation of -14069.24 from 1000 bootstrap replicates). Genomic DNA was isolated from the encysted metacercariae in the hepatopancreas of parasitised shore crabs across two locations (prefix: Apr (April) / Jun (June) / Aug (August), prefix: Mum (Mumbles Head) / Ox (Oxwich Bay) (GenBank: PQ314574 – PQ314610). Reference nucleotide sequences for *Microphallus* sp. from various intermediate hosts were retrieved from GenBank. The tree was rooted using *M. basodactylophallus* (OR457724.1). Sequences are highlighted according to species, *M. similis* (orange), *M. primas* (green).

Chapter Four

Discussion

4.1 Summary of findings

Digenean trematode parasites, including putative *M. similis* and *M. primas*, were observed in the hepatopancreas of shore crab populations from Mumbles Head and Oxwich Bay. *M. similis* appeared to maintain a consistent high prevalence at both locations, whereas *M. primas* was only found as a co-infection with *M. similis*, exhibiting a lower prevalence and infection intensity from June onwards. Microscopic examinations revealed no indication of host immune response (i.e. a lack of melanisation of cysts). At Oxwich Bay, the size and coloration of shore crabs were correlated with the presence of trematode parasites, while no environmental or biometric factors were associated with trematode presence at Mumbles Head. Phylogenetic analysis indicated a clear genetic distinction between *M. primas* and *M. similis*, with signs of phylogenetic diversity within *M. primas* across different months. The number of parasites per crab was significantly higher at Mumbles Head compared to Oxwich Bay, with larger crabs found to harbour a greater number of trematode metacercariae. Finally, encysted metacercariae in the hepatopancreas of crabs from Mumbles Head were significantly larger than those from Oxwich Bay.

4.2 Temporal changes in trematode appearance and prevalence

Despite both digenean trematode species *M. similis* and *M. primas* having been previously identified in shore crabs across U.K. coastlines (Stentiford and Feist 2005; Davies et al. 2022), research focusing on seasonal variations in prevalence within this secondary intermediate host remains absent. Based on the finding of this study, *M. similis* maintains a constant high level of parasite presence within shore crab populations from Mumbles and Oxwich from February to August. In contrast, *M. primas* only appeared and at a low prevalence from June onwards. These potential temporal changes in parasite presence may be due to factors including: (1) the migration of a population of crabs harbouring both *M. similis* and *M. primas* into the intertidal area between June and August, (2) an insufficient number of littorinid first intermediate host species to facilitate *M. primas* transmission in February and April, and (3) species variation in cercariae release which may also be influenced by environmental factors such as temperature. Davies et al. (2022) also identified two distinct forms of trematode metacercariae within the hepatopancreas of shore crabs from Swansea, U.K. using a histopathological approach. Although molecular techniques were not employed for species identification, the morphology of one type of encysted metacercaria closely resembles that of *M. primas*, as described in this study. This finding not only confirms the previous presence of *M. primas* in shore crabs from Swansea, U.K., but also demonstrates that co-infections

with these two species of *Microphallus* are not unique at these locations. The prior identification of *M. primas* in 2019 – 2020 suggests that it is not a more recent introduction to the Swansea coastline.

The disparity between the prevalence of *M. similis* and *M. primas* is likely to be associated with the habitat preference and population density of their respective first intermediate hosts. *M. similis* is known to have numerous first intermediate hosts, some of which have been reported at both Mumbles and Oxwich (Dixon and Pollard 1985; Warwick et al. 1990). However, the only documented first intermediate host of *M. primas* is the mudsnail (*Peringia ulvae*). Although *P. ulvae* has been observed along coastlines within the Bristol Channel (Mackie et al. 2007), it is primarily located on their preferred habitats of mudflats and estuaries (i.e. not rocky shores such as those studied here). Stentiford & Feist (2005) noted a high prevalence of *M. primas* in the hepatopancreas and gills of shore crabs sampled from Southampton Water and Alde estuaries, U.K. Similarly, Pina et al. (2011a), reported high a prevalence of *M. primas* in the hepatopancreas of shore crabs from Aveiro Estuary in Portugal. This suggests the low prevalence of *M. primas* in this present study may be linked to the choice of collection site, in that intertidal rocky shores do not support a sufficient population of *P. ulvae* to facilitate the high levels of parasite transmission observed in estuarine environments.

The presence of *M. primas* at both Mumbles and Oxwich sites beginning in June may be related to species-specific variations in cercariae release, potentially driven by environmental cues. McCarthy et al. (2002) noted distinct patterns in the release and behaviour of cercariae among species; specifically, *M. similis* produced fewer but larger and more robustly swimming cercariae that gravitated towards darker waters, unlike other microphallid species. These observations suggest that factors such as light and the specific characteristics of cercariae release and movement could play critical roles in the successful localization of a viable hosts. It has been well documented that temperature also has a significant effect on the release and survival of trematode cercariae, with the cercarial release of some trematode species increasing with temperature (Poulin 2006; Koprivnikar and Poulin 2009b; Rosen et al. 2018). This suggests that the abrupt appearance of *M. primas* may have been the direct cause of increasing summer temperatures in the intertidal zone. However, without a long-term comparative study of *M. similis* and *M. primas*, it remains uncertain whether these mechanisms are solely responsible for the inconsistent presence of *M. primas*, and that it is most likely to be influenced by a combination of host-parasite and environmental factors.

4.3 Effects of crab size and morphology on parasite prevalence

In the current study, size was found to be a significant factor in the prevalence of digenean parasites at Oxwich Bay only. This finding is consistent with previous research (e.g., Koga 2008;

Koehler and Poulin 2010; Blakeslee et al. 2015), which suggests that larger crabs, due to their longer exposure time, tend to accumulate more parasites and thus show higher parasitisation rates. However, while larger crabs naturally have more time to become parasitised, it is possible chemical cues may be involved. Hass (2003) reviewed the significance of host chemical cues in cercariae host-finding behaviour. Given that larger *C. maenas* likely emit stronger chemical cues, they may attract more trematode cercariae, thereby experiencing higher levels of parasite presence. An alternative explanation for the higher parasite prevalence in larger crabs is trematode-induced gigantism (Gorbushin 2009; Mouritsen & Jensen 1994). This phenomenon has been well-documented in gastropods, namely *Peringia ulvae,* whereby trematode-infected individuals exhibited increased growth rates compared to uninfected controls (Mouritsen & Jensen 1994). While this hypothesis may provide a plausible explanation for the observed size-parasite relationship in this present study, there is currently no evidence to suggest that trematode encystation induces a similar growth response in shore crabs. The absence of this consistently observed finding in shore crabs from Mumbles could be deemed anomalous. It is probable that the disparities in size (CW) of the crab populations sampled from Mumbles and Oxwich may contribute to the lack of observable relationship.

Coloration was also found to be a significant factor influencing the prevalence of digenean trematode parasites at Oxwich. Specifically, *C. maenas* with a yellow ventral surface were significantly less likely to be encysted with trematode metacercariae. Coloration in crabs is known to be indicative of the intermoult phase; yellow phase *C. maenas* typically represent a stage where the crabs are neither newly moulted nor soon to moult again (Styrishave et al. 2004). Although changes in exoskeletal coloration can reflect a crustacean's immune response to diseases such as shell disease (Lee & Söderhäll, 2002), colouration alone is unlikely to significantly affect trematode prevalence in shore crab populations due to it having no known direct impact on immune functionality. A more plausible explanation for the observed trend is the low sample size of yellow-phase *C. maenas* at this location, which has resulted in this apparent association.

4.4 Shore crab cellular immune responses to trematode infections

A recurring theme in research is the localised tissue damage caused by metacercariae (Robaldo et al. 1999; Díaz et al. 2004; Saville and Irwin 2005; Chubb et al. 2010). Robaldo et al. (1999) documented structural damage and necrosis of tubules in the hepatopancreas caused by *Microphallus* spp. encysted metacercariae. However, in the current study, neither apparent structural damage nor a host immune response to *M. similis* or *M. primas* metacercariae was observed. Similarly, Stentiford & Feist (2005) reported no damage to the hepatopancreas in heavily infected *C. maenas* by *M. primas*, although they noted a rather limited host immune response, characterised by a

thin layer of flattened haemocytes surrounding the encysted parasites but without any evidence of melanisation (a key indicator of a sustained encapsulation (i.e. immune) response). The histological images of encysted metacercariae in shore crabs by Davies et al. (2022) also failed to observe any evidence of an encapsulation response. Because it is thought that metacercariae in shore crabs have little effect on the survival, behaviour and physiology of their hosts (e.g. Blakeslee et al., 2015), it could be argued that eliciting a strong host response would be wasteful in terms of energy use as the parasites do little damage, if indeed any. The mechanisms potentially employed by cercariae to avoid/suppress the immune system are also unknown but worth further investigation.

4.5 The effects of shore crab size on parasite intensity

A notable finding in this study was that the number of encysted metacercariae per crab increased with crab size. This observation is consistent with prior research conducted at Adams Point, Durham, USA, where larger *C. maenas* were found to harbour higher numbers of *M. similis* metacercariae (Blakeslee et al., 2015). As previously discussed, it is likely that larger individuals can sustain higher-intensity infections due to their expansive haemocoel and prolonged exposure time, coupled with stronger chemical cues that attract more cercariae (Blakeslee et al., 2015). Additionally, Blakeslee et al. (2015) suggested that infection intensity may also be influenced by cercarial aggregatory behaviours. Specifically, larger crabs with higher infection intensities may attract more parasites due to the chemical signals released by the encysted metacercariae themselves. However, researchers highlighted that this is an unlikely mechanism due to higher intensity infections leading to smaller metacercariae and lower fecundity in adult worms (Blakeslee et al., 2015).

4.6 Metacercariae size variation

The size of digenean trematode metacercariae varies between species, often showing intraspecific variation due to different infection times and/or competition for space and resources within the host (Saldanha et al. 2009). Consistent with previous studies on *M. similis* (McCarthy et al., 2002) and *M. primas* (Saville & Irwin, 2005), the sizes of trematode metacercariae observed in this present study align with these previous findings. Fredensborg & Poulin (2005) highlighted that the size of metacercariae is density-dependent; notably, higher intensity trematode infections are typically correlated with smaller-sized metacercariae. Based on these previous findings, it would be expected that shore crabs from Mumbles, given their higher infection intensities, are more likely to host smaller *M. similis* metacercariae. However, contrary to expectations, *M. similis* metacercariae isolated from

crabs at Mumbles were significantly larger than those from hosts at Oxwich Bay. The findings also are a reminder that cyst size is not a reliable taxonomic tool for species identification.

4.7 Phylogenetic analysis

Phylogenetic reconstructions revealed little variation in the ecotype diversity of *M. similis* sequences between the two locations (Mumbles and Oxwich) and across different months. The majority of *M. similis* sequences clustered into a well-supported monophyletic clade, indicating genetic homogeneity within this species. In contrast, *M. primas* sequences exhibited some potential genetic variation, with June and August samples forming distinct clades. Whether these could represent the presence of more than one species is unclear, however, care should be taken in making conclusions based on the small number of samples examined and with only one primer set. Despite extensive research on the taxonomic diversity of digenean trematodes, studies specifically examining *M. similis* and *M. primas* in their crustacean hosts remain limited. Galaktionov et al. (2012) provided evidence supporting *M. similis* as a sister taxon to microphallids within the '*pygmaeus*' group, suggesting a shared common ancestry.

4.8 Final comments and limitations of this study

The current study was limited in terms of the sample sizes and timescale by the confines of time and resource availability. In terms of timescale, taking further samples throughout later months of the year (e.g. October – December) would have strengthened the conclusions of temporal changes associated with the late appearance of *M. primas.* If temperature and/or season was a driver of this occurrence, then later samples into the autumn and winter would be predicted to show the absence of this species. A further limitation relates to sample size that was linked to the amount of time needed to collect and process the crabs. While high levels of parasitisation were observed, some of the analyses would have been strengthened by larger sample sizes. The original brief of the project was to determine what species of trematodes infect shore crabs at the two sites using a primer set designed for taxonomic status. There is some suggestion from the results of polymorphism but the primers used cannot be easily employed to determine the level of single nucleotide polymorphism. Finally, while the original aim of this study was to examine the presence of digeneans in crabs only, it would have been pertinent to determine the distribution and parasitisation of the first intermediate hosts of *M. similis* including periwinkles such as *L. saxatilis* that are present in both study sites. A survey of the mudsnail (*P. ulvae*) would also have given insights into the possibility of these acting as first intermediate hosts for *M. primas*.

5.1 Conclusions

This study showed that digenean trematode parasites are present in shore crab populations across intertidal rocky shores around Swansea Gower. The high prevalence of *M. similis* throughout the duration of this study suggests that it is the dominant trematode species in these environments, likely benefiting from the abundance of first intermediate hosts. The association with crab size and trematode presence and intensity indicates that larger, older crabs harbour more parasites due to their prolonged exposure time, also noted by other studies. Phylogenetic analysis showed distinct clades for *M. similis* and *M. primas*. Cysts sizes were consistent with previous research, however, no association with infection intensity and size was identified. Overall, this study highlights the need for long-term research to identify seasonal variations, especially for *M. primas*, and potential environmental drivers responsible for the prevalence of these parasites.

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Chapter Six

Appendix

6.1 Abbreviations

CW: Carapace width

PCR: Polymerase chain reaction

rRNA: Ribosomal ribonucleic acid

DNA: Deoxyribonucleic acid

PITT: Parasite increased trophic transmission

KCL: Potassium chloride

NaCL: Sodium chloride

BLAST: Basic Local Alignment Search Tool

NCBI: National Centre for Biotechnology Information

SE: Standard Error

Table A1. Summary of biometric data for shore crabs (*Carcinus maenas*) surveyed between April and August.

Site(s)	Month	No. of crabs surveyed	Mean CW & (range) mm	M:F ratio	Epibionts (%)	Limb loss (%)	Shell Disease (%)	Spirorbis (%)	Colour morphs (G:Y:R)
Mumbles	April	30	47.9 (29 – 61)	13:17	3.3	20.0	13.3	6.7	1:1:28
"	June	24	45.6 (22 – 58)	10:14	16.7	29.2	16.7	0	6:0:18
"	August	21	32.5 (17 – 56)	15:6	14.3	33.3	9.5	4.8	17:2:2
Oxwich	April	30	33.9 (18 – 53)	15:15	6.7	13.3	3.3	0	9:0:21
"	June	28	37 (20 – 61)	15:13	25	35.7	3.6	0	17:2:9
"	August	30	36.5 (22 – 57)	18:12	10.0	20	0	3.3	18:6:6

Table A2. Full models used in order to predict response variable of presence of digenean trematodes before reduction. Asterisk denotes significance ($\alpha \leq 0.05$).

	Parameter	**Estimate (slope)**	**p**
Model 1 (Combined Population)			
TremPres ~ Location + Month + Sex + Fouling + Carapace.Width + Colour + Hemo.col	Location(Oxwich)	-0.90259	0.0756
AIC: 173.9	Month(June)	-0.89087	0.1355
	Month(August)	-0.42863	0.5039
	Sex(Male)	-0.51520	0.2662
	Fouling	1.70612	0.1568
	Colour(Red)	-0.23186	0.6890
	Colour(Yellow)	-1.05689	0.2372
	Carapace Width	0.05987	0.0232 *
	Pigment Loss	-0.06769	0.9171
	Haemolymph Opacity	-1.26108	0.1778
Model 2 (Mumbles)			
TremPres ~ Month + Sex + Fouling + Carapace.Width + Colour + Hemo.col + Pigment.Loss	Month(June)	-0.26906	0.764
	Month(August)	-2.11728	0.221
	Sex(Male)	-0.09234	0.904
	Fouling	0.71724	0.627
AIC: 76.2	Carapace Width	0.01891	0.670
	Colour(Red)	-2.16780	0.154
	Colour(Yellow)	15.11622	0.994
	Haemolymph Opacity	-1.00016	0.297
	Pigment Loss	-0.50711	0.523
Model 3 (Oxwich)			
TremPres ~ Month + Sex + Fouling + Carapace.Width + Colour + Hemo.col + Pigment.Loss	Month(June)	-1.23026	0.12898
	Month(August)	-0.24251	0.77545
	Sex(Male)	-0.53551	0.36559
	Fouling	30.57478	0.99237
AIC: 96.5	Carapace Width	0.11640	0.00716 **
	Colour(Red)	0.22229	0.77555
	Colour(Yellow)	-2.88777	0.02389 *
	Haemolymph Opacity	-16.95597	0.99402
	Pigment Loss	2.02974	0.20562

Table A3. Accession numbers, deposited in GenBank, and corresponding sampling numbers for all trematode-positive animals successfully sequenced from this study, and used in phylogenetic tree (Figure 11).

GenBankID	Seq	Sample	Primers	Sample Type
PQ314574	Seq1	Apr_Mum_04	LSU-5/LSU-1500R	Metacercariae
PQ314575	Seq2	Apr_Mum_07	LSU-5/LSU-1500R	Metacercariae
PQ314576	Seq3	Apr_Mum_25	LSU-5/LSU-1500R	Metacercariae
PQ314577	Seq4	Apr_Ox_09	LSU-5/LSU-1500R	Metacercariae
PQ314578	Seq5	Apr_Ox_14	LSU-5/LSU-1500R	Metacercariae
PQ314583	Seq6	Jun_Mum_01	LSU-5/LSU-1500R	Metacercariae
PQ314584	Seq7	Jun_Mum_02	LSU-5/LSU-1500R	Metacercariae
PQ314585	Seq8	Jun_Mum_03	LSU-5/LSU-1500R	Metacercariae
PQ314586	Seq9	Jun_Mum_04a	LSU-5/LSU-1500R	Metacercariae
PQ314588	Seq11	Jun_Mum_06	LSU-5/LSU-1500R	Metacercariae
PQ314589	Seq12	Jun_Mum_15	LSU-5/LSU-1500R	Metacercariae
PQ314590	Seq13	Jun_Mum_18	LSU-5/LSU-1500R	Metacercariae
PQ314591	Seq14	Jun_Mum_22	LSU-5/LSU-1500R	Metacercariae
PQ314592	Seq15	Jun_Mum_12	LSU-5/LSU-1500R	Metacercariae
PQ314598	Seq16	Jun_Ox_01	LSU-5/LSU-1500R	Metacercariae
PQ314599	Seq17	Jun_Ox_04	LSU-5/LSU-1500R	Metacercariae
PQ314600	Seq18	Jun_Ox_06	LSU-5/LSU-1500R	Metacercariae
PQ314601	Seq19	Jun_Ox_12	LSU-5/LSU-1500R	Metacercariae
PQ314602	Seq20	Jun_Ox_13	LSU-5/LSU-1500R	Metacercariae
PQ314603	Seq21	Jun_Ox_15	LSU-5/LSU-1500R	Metacercariae
PQ314604	Seq22	Jun_Ox_17	LSU-5/LSU-1500R	Metacercariae
PQ314605	Seq23	Jun_Ox_21	LSU-5/LSU-1500R	Metacercariae
PQ314607	Seq25	Aug_Mum_14a	LSU-5/LSU-1500R	Metacercariae
PQ314608	Seq26	Aug_Mum_14b	LSU-5/LSU-1500R	Metacercariae
PQ314609	Seq27	Aug_Mum_15	LSU-5/LSU-1500R	Metacercariae
PQ314610	Seq28	Aug_Ox_15	LSU-5/LSU-1500R	Metacercariae

Table A4. Reference accession numbers acquired from GenBank used in phylogenetic tree (Figure 13).

GenBankID	Trematode Species	Host Species
HM584136.1	*Microphallus similis*	*Larus schistisagus*

MG783587.1	*Microphallus ochotensis*	*Somateria mollissima*
MG783588.1	*Microphallus ochotensis*	*Falsicingula kurilensis*
OR457724.1	*Microphallus basodactylophallus*	*Eudocimus albus*
AY220628.1	*Microphallus basodactylophallus*	*Oryzomys palustris*
AY220626.1	*Microphallus abortivus*	*Peringia ulvae*
AY220627.1	*Microphallus primas*	*Peringia ulvae*
HM001304.1	*Microphallus primas*	*Carcinus maenas*
ON036094.1	*Parabascus lepidotus*	
ON036091.1	*Parabascus lepidotus*	
HM584138.1	*Microphallus similis*	*Larus schistisagus*
HM584137.1	*Microphallus similis*	*Larus schistisagus*
AY220625.1	*Microphallus similis*	*Larus schistisagus*
KT355822.1	*Microphallus minutus*	*Cherax dispar*
KT355823.1	*Microphallus minutus*	*Posticobia brazieri*
HM584122.1	*Microphallus piriformes*	*Littorina saxatilis*
HM584123.1	*Microphallus piriformes*	*Littorina saxatilis*
HM584124.1	*Microphallus calidris*	*Littorina sitkana*
HM584125.1	*Microphallus calidris*	*Littorina sitkana*
HM584139.1	*Microphallus triangulatus*	*Somateria mollissima*
HM584128.1	*Microphallus triangulatus*	*Littorina saxatilis*
HM584126.1	*Microphallus pseudopygmaeus*	*Onoba aculeus*
HM584127.1	*Microphallus pseudopygmaeus*	*Littorina saxatilis*
HM584133.1	*Microphallus pygmaeus*	*Littorina saxatilis*
HM584134.1	*Microphallus pygmaeus*	*Littorina saxatilis*
MK422193.1	*Microphallus primas*	*Carcinus maenas*
HM001303.1	*Microphallus primas*	*Carcinus maenas*
MT025349.1	*Microphallus primas*	*Carcinus maenas*
MT025354.1	*Microphallus primas*	*Carcinus maenas*
OP125524.1	*Microphallus primas*	
MT025339.1	*Microphallus primas*	*Carcinus maenas*
AJ287541.1	*Microphallus primas*	*Carcinus maenas*
OP125522.1	*Microphallus primas*	
OP125523.1	*Microphallus primas*	
MT025353.1	*Microphallus primas*	*Carcinus maenas*
MT025355.1	*Microphallus primas*	*Carcinus maenas*
OP125525.1	*Microphallus primas*	

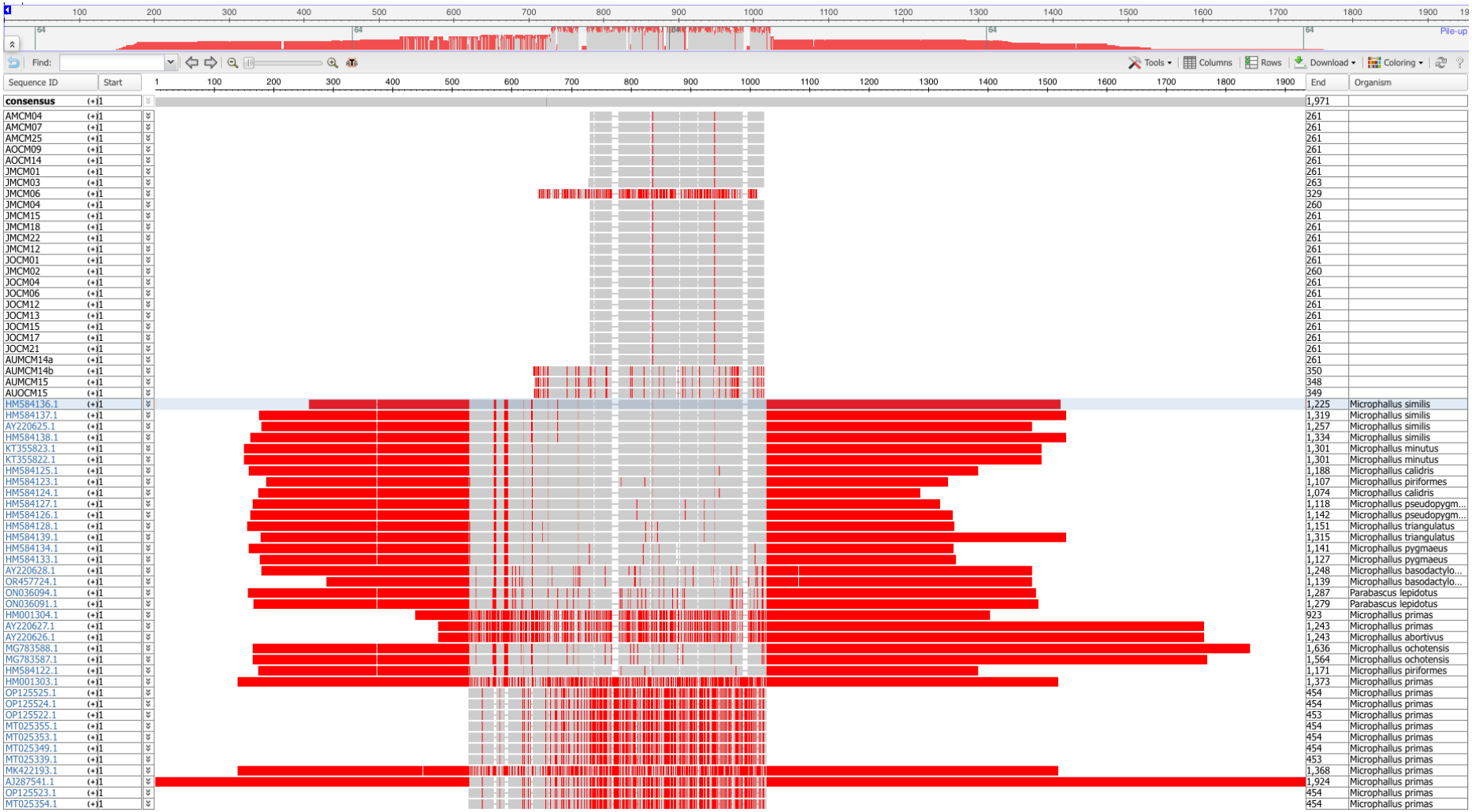

Figure A1. Multiple sequence alignment of sequences used to create phylogenetic tree (Figure 13).

Table A5. Statement of expenditure

Student Name: Alex Bedford
Student Number: [redacted]
Project Title: Digenean parasites of shore crabs on Swansea Gower

Category	Item	Description	Cost
Travel	Parking Ticket	Mumbles pier parking	£2.50
Travel	Parking Ticket	Mumbles pier parking	£3.00
Travel	Parking Ticket	Mumbles pier parking	£3.00
Travel	Parking Ticket	Oxwich parking	£5.00
Travel	Parking Ticket	Oxwich parking	£7.00
Travel	Parking Ticket	Oxwich parking	£7.00
*including VAT and delivery where applicable			

I hereby certify that the information is true and correct to the best of my knowledge.

[redacted]
Signature (Supervisor)

[redacted]
Signature (Student)

Table A6. Statement of contributions

Contributor Role	Role Definition
Conceptualisation	A.F.R, C.E.D
Data Curation	A.B, C.G, A.F.R, C.E.D
Formal Analysis	C.E.D, A.B, G.C
Funding Acquisition	A.F.R, C.E.D
Investigation	A.B, C.G, A.F.R, C.E.D
Methodology	A.B, C.G, A.F.R, C.E.D
Project Administration	A.F.R, C.E.D
Resources	A.F.R, C.E.D
Software	C.E.D, A.B, G.C
Supervision	A.F.R, C.E.D
Validation	A.F.R, C.E.D
Visualisation	A.B, C.G, A.F.R, C.E.D
Writing – Original draft preparation	A.B, A.F.R, C.E.D
Writing – Review & Editing	A.B, A.F.R, C.E.D

6.2 R-Script

```
rm(list=ls(all=TRUE)) # Removes everything from R (all objects) - a good thing to do if you are starting fresh work
graphics.off()        # Removes any graphics or graphic windows that may exist - again a good thing to do if you are starting to work fresh

setwd()

install.packages("quantreg")
install.packages("SparseM")
install.packages("Rearrangement")
install.packages("reshape")
install.packages("MuMIn") #only needed if we want to use DREDGE
library(MASS)
library(Rearrangement)
library(reshape)
library(MuMIn)

disease.data<-read.exl(file.choose())
disease.data <- Disease_data
names(disease.data)
head(disease.data)
str(disease.data) # gives full structure of dataset
attach(disease.data)
detach(disease.data)
attach(disease.data)

### Factoring catagorical variables
TremPres <- factor(TremPres)
Carapace.Width <- factor(Carapace.Width)
Sex<- factor(Sex)
Limb.Loss <- factor(LimbLoss)
Fouling<- factor(Fouling)
Colour<- factor(Colour)
Location<- factor(Location)
HemoCol<- factor(Hemo.col)
Tube.Hepato.g <- factor(Tube.Hepato.g)
Crab.Weight.g <- factor(Crab.Weight.g)
Pigment.Loss <- factor(Pigment.Loss)
Month <- factor(Month)
Parasite/g <- factor(Parasite/g)
## note: categorical variables are automatically taken as factors
# when written into models - however continuous variables need to be specified as factors, e.g. CW

disease.data.Mumbles<-subset(disease.data,Location=='Mumbles') #subsetting for location for later models
disease.data.Oxwich<-subset(disease.data,Location=='Oxwich')
```

```
#FULL MODEL 1 INC Pigment.Loss

Trem_All <- glm(TremPres ~ Location + Month + Sex + Fouling + Colour + Carapace.Width
+ Pigment.Loss + Hemo.col, data=disease.data, family=binomial(link = "logit"),
na.action=na.exclude)
summary.lm (Trem_All)
extractAIC (Trem_All)

stres<- (Trem_All$residuals - mean(Trem_All$residuals))/sd(Trem_All$residuals)
hist(stres)
plot(stres ~ Trem_All$fitted.values)
plot(Trem_All)



drop1(Trem_All, test="Chisq") #remove Pigment.Loss

Trem_reduced1 <- glm(TremPres ~ Location + Month + Sex + Fouling + Carapace.Width +
Colour + Hemo.col , data=disease.data, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Trem_reduced1)
extractAIC(Trem_reduced1)

drop1(Trem_reduced1, test="Chisq") # remove Colour

Trem_reduced2 <- glm(TremPres ~ Location + Month + Sex + Fouling + Carapace.Width +
Hemo.col, data=disease.data, family=binomial(link = "logit"), na.action=na.exclude)
summary (Trem_reduced2)

drop1(Trem_reduced2, test="Chisq") # remove Limb Loss

Trem_reduced3 <- glm(TremPres ~ Location + Month + Sex + Fouling + Carapace.Width +
Hemo.col, data=disease.data, family=binomial(link = "logit"), na.action=na.exclude)
summary (Trem_reduced3)

drop1(Trem_reduced3, test="Chisq") #remove month

Trem_reduced4 <- glm(TremPres~ Location + Sex + Fouling + Carapace.Width + Hemo.col,
data=disease.data, family=binomial(link = "logit"), na.action=na.exclude)
summary (Trem_reduced4)

drop1(Trem_reduced4, test="Chisq") #remove Haemolymph Colour

Trem_reduced5 <- glm(TremPres ~ Location + Sex + Fouling + Carapace.Width,
data=disease.data, family=binomial(link = "logit"), na.action=na.exclude)
summary (Trem_reduced5)

drop1(Trem_reduced5, test="Chisq") #remove Fouling
```

```
Trem_reduced6 <- glm(TremPres ~ Location + Sex + Carapace.Width, data=disease.data,
family=binomial(link = "logit"), na.action=na.exclude)
summary (Trem_reduced6)

str(Limb.Loss)

####SEPARATING BY LOCATION####

#FULL MUMBLES MODEL 1

Mumbles_Trem_ALL <- glm(TremPres ~ Month + Sex + Fouling + Carapace.Width +
Colour + Hemo.col + Pigment.Loss, data=disease.data.Mumbles, family=binomial(link =
"logit"), na.action=na.exclude)
summary (Mumbles_Trem_ALL)
extractAIC(Mumbles_Trem_ALL)

drop1(Mumbles_Trem_ALL, test="Chisq") #remove sex

Mumbles_Trem_reduced1 <- glm(TremPres ~ Month + Fouling + Carapace.Width + Colour
+ Hemo.col + Pigment.Loss, data=disease.data.Mumbles, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Mumbles_Trem_reduced1)

drop1(Mumbles_Trem_reduced1, test="Chisq") # remove Carapace Width

Mumbles_Trem_reduced2 <- glm(TremPres ~ Month + Fouling + Colour + Hemo.col +
Pigment.Loss, data=disease.data.Mumbles, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Mumbles_Trem_reduced2)

drop1(Mumbles_Trem_reduced2, test="Chisq") #remove Pigment Loss

Mumbles_Trem_reduced3 <- glm(TremPres ~ Month + Fouling + Colour + Hemo.col,
data=disease.data.Mumbles, family=binomial(link = "logit"), na.action=na.exclude)
summary (Mumbles_Trem_reduced3)

drop1(Mumbles_Trem_reduced3, test="Chisq") #remove fouling

Mumbles_Trem_reduced4 <- glm(TremPres ~  Month + Hemo.col + Colour,
data=disease.data.Mumbles, family=binomial(link = "logit"), na.action=na.exclude)
summary (Mumbles_Trem_reduced4)

drop1(Mumbles_Trem_reduced4, test="Chisq") #remove Hemocol

Mumbles_Trem_reduced5 <- glm(TremPres ~  Month + Colour, data=disease.data.Mumbles,
family=binomial(link = "logit"), na.action=na.exclude)
summary (Mumbles_Trem_reduced5)

drop1(Mumbles_Trem_reduced5, test="Chisq")
```

```
#FULL OXWICH MODEL 1

Oxwich_Trem_ALL <- glm(TremPres ~ Month + Sex + Fouling + Carapace.Width + Colour
+ Hemo.col + Pigment.Loss, data=disease.data.Oxwich, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Oxwich_Trem_ALL)


drop1(Oxwich_Trem_ALL, test="Chisq") #remove Sex

Oxwich_Trem_reduced1 <- glm(TremPres ~ Month + Fouling + Carapace.Width + Colour +
Hemo.col + Pigment.Loss, data=disease.data.Oxwich, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Oxwich_Trem_reduced1)

drop1(Oxwich_Trem_reduced1, test="Chisq") # remove Month

Oxwich_Trem_reduced2 <- glm(TremPres ~ Fouling + Carapace.Width + Colour + Hemo.col
+ Pigment.Loss, data=disease.data.Oxwich, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Oxwich_Trem_reduced2)

drop1(Oxwich_Trem_reduced2, test="Chisq") #remove Hemolymph color

Oxwich_Trem_reduced3 <- glm(TremPres ~  Fouling + Carapace.Width + Colour +
Pigment.Loss, data=disease.data.Oxwich, family=binomial(link = "logit"),
na.action=na.exclude)
summary (Oxwich_Trem_reduced3)

drop1(Oxwich_Trem_reduced3, test="Chisq") #remove Pigment loss

Oxwich_Trem_reduced4 <- glm(TremPres ~  Fouling + Carapace.Width + Colour,
data=disease.data.Oxwich, family=binomial(link = "logit"), na.action=na.exclude)
summary (Oxwich_Trem_reduced4)

drop1(Oxwich_Trem_reduced4, test="Chisq") #remove Fouling

Oxwich_Trem_reduced5 <- glm(TremPres ~ Carapace.Width + Colour,
data=disease.data.Oxwich, family=binomial(link = "logit"), na.action=na.exclude)
summary (Oxwich_Trem_reduced5)

drop1(Mumbles_Trem_reduced5, test="Chisq")

glm_neg_load <- glm.nb(Parasite.count ~ Location + Month + Sex + Carapace.Width +
Hemo.col,data = disease.data.load1)
summary(glm_neg_load)
vif(glm_neg_load)
```

6.3 Health and safety documents

6.3.1 Aquarium

Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name.....Alexander Bedford..... **Signature** [redacted] **date** 01/02/2024

Supervisor* .....Prof Andrew Rowley..... **Signature** [redacted] **date** 01/02/2024

Activity title .....Digenean parasites of shore crabs..... **Base location (room no.)** .....

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

University Activity Serial # (enter Employee No. or STUREC No. [redacted]

Start date of activity (cannot predate signature dates) .....01/02/2024.....

End date of activity (or 'on going').....30/09/2024.....

Level of worker (delete as applicable).....Postgraduate 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
Aquarium	01/02/24

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 N/A if not applicable)

Protocol #	**Title:** Crab Storing
Associated Protocols #**.....**	**Description:**

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

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
N/A	N/A	N/A	N/A	N/A

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) :-

Storage conditions and maximum duration :-

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

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

Identify other control measures (circle or delete) latex/nitrile/heavy gloves; screens; full face mask; dust mask; protective shoes; spillage tray; ear-defenders; other (state)

Justification and controls for any work outside normal hours

Emergency procedures (e.g. spillage clearance; communication methods)

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 Alexander Bedford [redacted]

Name & counter-signature of supervisor Prof Andrew Rowley [redacted] *Date* 01/02/24

Date of first reassessment Frequency of reassessments

6.3.2 Benthos Laboratory

V1.1 2023

Risk Assessment for Teaching and Research Activities*

Swansea University; FSE: Biosciences

Name Alexander Bedford **Signature** [redacted] **date** 01/02/2024

Supervisor* Prof Andrew Rowley **Signature** [redacted] **date** 01/02/2024

Activity title Digenean parasites of shore crabs **Base location (room no.)**

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOD or their nominee)

University Activity Serial # (enter Employee No. or Student No.) [redacted]

Start date of activity (cannot predate signature dates) 01/02/2024

End date of activity (or 'on going') 30/09/2024

Level of worker (choose from the list below) M. Res

UG, MSc, M.Res, M.Phil/PhD, RA/Postdoc, technician, administration, academic staff, visitor, other (state)

Ethics approval number 2 2024 8411 7714

Approval obtained for Biological Hazards and/or GMO Safety Assessment by SU? Yes/not applicable

Is your project: (circle the appropriate choice A-D)

A. Laboratory-based only (i.e. you **never** work in the field)
B. Field **AND** laboratory-based
C. Field-only based (i.e. you do not have an allocated laboratory space and **never** work in a laboratory)
D. Desk based (i.e. no field or laboratory base. i.e. you are only allocated office space [if you are a PhD or research member of staff])

For **category A** complete this Risk Assessment template and associated laboratory protocols, and a Training Record form.
For **category B** complete this Risk Assessment template and associated laboratory protocols, a Training Record form, AND either complete the FSE on-line Field Risk Assessment (**for UG, MSc**) or the relevant University-template form (i.e. Red Form- Off Campus Activities & Risk Assessment Form) (**for MRes, PhD, all staff, visitors**)
For **category C** complete this Risk Assessment template (but not the protocol sheets) and the relevant on-line FSE field risk assessment or University-template forms (see B above for details) and complete a Training Record
For **category D** complete the Training Record template **and** this front page.

***N.B. All staff, visitors and students must have risk assessments for their studies in the University. No work can commence until these have been completed. They must be always available for inspection. Some of these may be paper-based but others can be stored electronically.**

1

Summary of laboratory and/or field protocols used; protocol sheets to be appended and updated as necessary

#	Title	1st Assessment Date	Frequency of re-assessment
1	Protocol 1	02/02/24	N/A
2			
3			
4			
5			
6			
7			
8			
9			
10			

Reassessment - the first reassessment must be undertaken as soon as possible after the first time the protocol has been undertaken in order to identify any unforeseen hazards. After this first reassessment, the protocol should be reassessed every 6-12m. The protocol must be reassessed immediately if new knowledge on the chemical hazards becomes available.

Protocol Risk Assessment Form (Laboratory-only)

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

Protocol #	**Title:** Crab dissection
Associated Protocols #.....	**Location and local rules** *In addition to Good Laboratory Practice, identify any local rules that apply (specific risks and control measures for work in this environment).* W043
Description of the protocol: Crab bleeding, dissection, and recording biometric data, sacrifice crabs with 1ml KCL	
Additional risks and control measures specific to this protocol: *In addition to the local rules, identify the risks associated with use of equipment (e.g. autoclaves, centrifuges), other mechanical and electrical hazards AND control measures.* **Note chemical hazards are summarised below and any biological hazards should be identified in a separate Biological Risk Assessment form.*	
Who or what may be harmed? ☒ Staff/ PG student carrying out the activity ☐ Contractors ☐ Visitors ☐ Cleaners ☐ Maintenance staff ☐ UG student carrying out activity ☐ Other staff/ students in the vicinity	**Vulnerable groups present:** ☐ U18/ U16 ☐ New or expectant mother ☐ Other: ☐ **Environment** (via release to air/water/ground, or incorrect disposal)

PROTOCOL RISK MANAGEMENT

Secondary Containment (of protocol): e.g. open bench/fume hood/special **N/A**	
Measures taken to eliminate or substitute/reduce: e.g. using less hazardous, less volume of chemicals N/A	
Personal Protective Equipment and all specific control measures Include a full description e.g. latex/nitrile/heavy gloves; safety glasses, screens; full face mask; dust mask; protective shoes; spillage tray; ear-defenders; other (state)	
Emergency procedures (include first aid, fire, spillage, communication methods) N.B. full emergency plans for each chemical are detailed in individual Chemical data Sheets N/A	
Is exposure monitoring required? Yes (give details) or No No	**Is health surveillance required?** Yes (give details) or No No
Justification and controls for any work outside normal hours (N.B. UG project students cannot work outside normal hours in a laboratory) No outside hours	
Supervision/training for worker (highlight) **N.B. All relevant training forms (e.g. for specific laboratories) should be completed** 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* ..... Alexander Bedford [redacted] ..... *Name & counter-signature of supervisor*..... Prof Andrew Rowley [redacted] ..... *Date*..... 01/02/2024	
Date of first reassessment	Frequency of reassessments

5

6.3.3 Molecular Laboratory

V1.1 2023

Risk Assessment for Teaching and Research Activities*

Swansea University; FSE: Biosciences

Name Alexander Bedford **Signature** [redacted] **date** 01/02/2024

Supervisor* Prof Andrew Rowley **Signature** [redacted] **date** 01/02/2024

Activity title Digenean parasites of shore crabs **Base location (room no.)**....

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOD or their nominee)

University Activity Serial # (enter Employee No. or Student No.) [redacted]

Start date of activity (cannot predate signature dates) 01/02/2024

End date of activity (or 'on going') 30/09/2024

Level of worker (choose from the list below) M. Res

UG, MSc, M.Res, M.Phil/PhD, RA/Postdoc, technician, administration, academic staff, visitor, other (state)

Ethics approval number 2 2024 8411 7714

Approval obtained for Biological Hazards and/or GMO Safety Assessment by SU? Yes/not applicable

Is your project: (circle the appropriate choice A-D)

A. Laboratory-based only (i.e. you **never** work in the field)
B. Field **AND** laboratory-based
C. Field-only based (i.e. you do not have an allocated laboratory space and **never** work in a laboratory)
D. Desk based (i.e. no field or laboratory base. i.e. you are only allocated office space [if you are a PhD or research member of staff])

For **category A** complete this Risk Assessment template and associated laboratory protocols, and a Training Record form.
For **category B** complete this Risk Assessment template and associated laboratory protocols, a Training Record form, AND either complete the FSE on-line Field Risk Assessment (**for UG, MSc**) or the relevant University-template form (i.e. Red Form- Off Campus Activities & Risk Assessment Form) (**for MRes, PhD, all staff, visitors**)
For **category C** complete this Risk Assessment template (but not the protocol sheets) and the relevant on-line FSE field risk assessment or University-template forms (see B above for details) and complete a Training Record
For **category D** complete the Training Record template **and** this front page.

***N.B. All staff, visitors and students must have risk assessments for their studies in the University. No work can commence until these have been completed. They must be always available for inspection. Some of these may be paper-based but others can be stored electronically.**

1

Summary of laboratory and/or field protocols used; protocol sheets to be appended and updated as necessary

#	Title	1st Assessment Date	Frequency of re-assessment
1	PCR (Amplification of DNA) and DNA visualisation via gel electrophoresis	17/04/24	N/A
2			
3			
4			
5			
6			
7			
8			
9			
10			

Reassessment - the first reassessment must be undertaken as soon as possible after the first time the protocol has been undertaken in order to identify any unforeseen hazards. After this first reassessment, the protocol should be reassessed every 6-12m. The protocol must be reassessed immediately if new knowledge on the chemical hazards becomes available.

Protocol Risk Assessment Form (Laboratory-only)

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

Protocol 2	**Title: PCR (Amplification of DNA) and DNA visualisation via gel electrophoresis**
Associated Protocols #.....	**Location and local rules** *In addition to Good Laboratory Practice, identify any local rules that apply (specific risks and control measures for work in this environment).* Shared molecular facility Wallace 131a Benthos Lab Wallace 043

Description of the protocol: PCR (Amplification of DNA) and DNA visualisation via gel electrophoresis

Additional risks and control measures specific to this protocol:

In addition to the local rules, identify the risks associated with use of equipment (e.g. autoclaves, centrifuges), other mechanical and electrical hazards AND control measures.
**Note chemical hazards are summarised below and any biological hazards should be identified in a separate Biological Risk Assessment form.*

Use of centrifuges, vortex, class 2 hood, UV light sterilization, bleach (for cleaning), PCR machine, gel tanks

Who or what may be harmed?	
☒ Staff/ PG student carrying out the activity ☐ Contractors ☐ Visitors ☐ Cleaners ☐ Maintenance staff ☐ UG student carrying out activity ☐ Other staff/ students in the vicinity	**Vulnerable groups present:** ☐ U18/ U16 ☐ New or expectant mother ☐ Other: ☐ **Environment** (via release to air/water/ground, or incorrect disposal)

A copy of each Chemical COSSH form should be readily available in the lab for use (e.g. in an emergency)								
Chemical Name (& Conc.) for chemicals to be used and generated	**GHS symbols** (SH, AT, H, C, Ex, F, O, Env, CG) All that are applicable.	**Skin/Eyes Group** (SA, SB, SC, SD, SE)	**Inhalation Group** (A,B,C,D,E)	**Quantity**	***In use* dustiness or volatility**	**Disposal**	**Primary containment & storage**	**Other comments:** **In use factors affecting exposure and special control measures** *(e.g. <15 mins duration/ frequency/ splash protection only/ hand immersion/ spraying)* **Safety/ environmental hazards (H2XX/H4XX)**
Master mix	N/A	SA	A	1 - Small	Low volatility	*SU chemical disposal*		
Primers	N/A	SA	A	1 - Small	Low volatility	*SU chemical disposal*		
TAE x1 & x10	Env, H	SA	A	1 - Small	Low volatility	*SU chemical disposal*		
Agarose	N/A	SA	A	1 - Small	Low volatility	*SU chemical disposal*		
Bleach	C, Env	SB	B	1 - Small	Low volatility	*SU chemical disposal*		

GHS symbols– SH (serious health hazard), AT (acute toxicity), H (health hazard), C (corrosive), Ex (explosive), F (flammable), O (oxidiser), Env (environment), CG (compressed gas). These should be obtained from chemical SDS documentation. See Appendix (hazard symbols).
Inhalation Group and Skin/Eyes Group- Hazard groups are classified as A/SA (least hazardous) to E/SE (most hazardous). See Appendix for hazard phrases associated with each group. Hazard phrases can be found on chemical SDS documentation.
Dustiness. Low (Pellet- does not break up), Medium (granular or crystalline), High (fine solid or light powder/dust)
Volatility. Low, medium, high, gas. Consider boiling point of liquid and operating temperature.
Disposal e.g. autoclaving of biohazard, SU chemical disposal
Primary containment: e.g. sealed flask, supplied vessel. **Storage:** e.g. secure chemical storage, fridge, freezer, general chemical storage

Note: A **specific DSEAR risk assessment** must be carried out if:

- The work activity involves the use or storage of **flammable, oxidising or corrosive gas cylinders.**
- The work activity is likely to create an **explosive atmosphere** even after the application of controls stated in the chemical risk assessment.
- The work activity involves the **use of explosives.**

Secondary Containment (of protocol): e.g. open bench/fume hood/special

Open bench and fume hood for larger volumes

Measures taken to eliminate or substitute/reduce: e.g. using less hazardous, less volume of chemicals

Using less hazardous, less volume of chemicals

Personal Protective Equipment and all specific control measures Include a full description e.g. latex/nitrile/heavy gloves; safety glasses, screens; full face mask; dust mask; protective shoes; spillage tray; ear-defenders; other (state)

Emergency procedures (include first aid, fire, spillage, communication methods) N.B. full emergency plans for each chemical are detailed in individual Chemical data Sheets

Individual chemical data sheets

Is exposure monitoring required? Yes (give details) or No	**Is health surveillance required?** Yes (give details) or No
No	No

Justification and controls for any work outside normal hours (N.B. UG project students cannot work outside normal hours in a laboratory)

No outside hours

Supervision/training for worker (highlight) **N.B. All relevant training forms (e.g. for specific laboratories) should be completed**

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 ..... Alexander Bedford [redacted] .....

Name & counter-signature of supervisor..... Prof Andrew Rowley [redacted] ..... *Date*..... 01/02/2024 .....

Date of first reassessment | Frequency of reassessments

Risk Assessment for Teaching and Research Activities*

Swansea University; FSE: Biosciences

Summary of laboratory and/or field protocols used; protocol sheets to be appended and updated as necessary

#	Title	1st Assessment Date	Frequency of re-assessment
1	DNA Extraction	17/04/24	N/A
2			
3			
4			
5			
6			
7			
8			
9			
10			

Reassessment - the first reassessment must be undertaken as soon as possible after the first time the protocol has been undertaken in order to identify any unforeseen hazards. After this first reassessment, the protocol should be reassessed every 6-12m. The protocol must be reassessed immediately if new knowledge on the chemical hazards becomes available.

D. Desk based (i.e. no field or laboratory base. i.e. you are only allocated office space [if you are a PhD or research member of staff])

For **category A** complete this Risk Assessment template and associated laboratory protocols, and a Training Record form.
For **category B** complete this Risk Assessment template and associated laboratory protocols, a Training Record form, AND either complete the FSE on-line Field Risk Assessment (**for UG, MSc**) or the relevant University-template form (i.e. Red Form- Off Campus Activities & Risk Assessment Form) (**for MRes, PhD, all staff, visitors**)
For **category C** complete this Risk Assessment template (but not the protocol sheets) and the relevant on-line FSE field risk assessment or University-template forms (see B above for details) and complete a Training Record
For **category D** complete the Training Record template **and** this front page.

***N.B. All staff, visitors and students must have risk assessments for their studies in the University. No work can commence until these have been completed. They must be always available for inspection. Some of these may be paper-based but others can be stored electronically.**

1

Protocol Risk Assessment Form (Laboratory-only)

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

Protocol 2	**Title: DNA Extraction**
Associated Protocols #.....	**Location and local rules** *In addition to Good Laboratory Practice, identify any local rules that apply (specific risks and control measures for work in this environment).* Shared molecular facility Wallace 131a Benthos Lab Wallace 043

Description of the protocol: DNA Extraction

Additional risks and control measures specific to this protocol:

In addition to the local rules, identify the risks associated with use of equipment (e.g. autoclaves, centrifuges), other mechanical and electrical hazards AND control measures.
**Note chemical hazards are summarised below and any biological hazards should be identified in a separate Biological Risk Assessment form.*

Use of centrifuges, vortex, class 2 hood, UV light sterilization, bleach (for cleaning), PCR machine, gel tanks

Who or what may be harmed?	
☒ Staff/ PG student carrying out the activity ☐ Contractors ☐ Visitors ☐ Cleaners ☐ Maintenance staff ☐ UG student carrying out activity ☐ Other staff/ students in the vicinity	**Vulnerable groups present:** ☐ U18/ U16 ☐ New or expectant mother ☐ Other: ☐ **Environment** (via release to air/water/ground, or incorrect disposal)

A copy of each Chemical COSSH form should be readily available in the lab for use (e.g. in an emergency)								
Chemical Name (& Conc.) for chemicals to be used and generated	**GHS symbols** (SH, AT, H, C, Ex, F, O, Env, CG) All that are applicable.	**Skin/Eyes Group** (SA, SB, SC, SD, SE)	**Inhalation Group** (A,B,C,D,E)	**Quantity**	***In use* dustiness or volatility**	**Disposal**	**Primary containment & storage**	**Other comments:** **In use factors affecting exposure and special control measures** *(e.g. <15 mins duration/ frequency/ splash protection only/ hand immersion/ spraying)* **Safety/ environmental hazards (H2XX/H4XX)**
Buffer AW2	N/A	SA	A	1 - Small	Low volatility	*SU chemical disposal*	*General Chemical Storage*	
Protenase K	SH	SE	E	1 - Small	High volatility	*SU chemical disposal*	*General Chemical Storage*	
Buffer ATL	N/A	SA	A	1 - Small	Low volatility	*SU chemical disposal*	*General Chemical Storage*	
Buffer AE	N/A	SA	A	1 - Small	Low volatility	*SU chemical disposal*	*General Chemical Storage*	
Buffer AW1	H	SB	B	1 - Small	Low volatility	*SU chemical disposal*	*General Chemical Storage*	
Buffer ATL	H	SE	E	1 - Small	Low volatility	*SU chemical disposal*	*General Chemical Storage*	
Bleach	C, Env	SB	B	1 – Small	Low volatility	*SU chemical disposal*	*General Chemical Storage*	
Ethanol	F	SB	A	1 - Small	High volatility	*SU chemical disposal*	*General Chemical Storage*	

GHS symbols– SH (serious health hazard), AT (acute toxicity), H (health hazard), C (corrosive), Ex (explosive), F (flammable), O (oxidiser), Env (environment), CG (compressed gas). These should be obtained from chemical SDS documentation. See Appendix (hazard symbols).
Inhalation Group and Skin/Eyes Group- Hazard groups are classified as A/SA (least hazardous) to E/SE (most hazardous). See Appendix for hazard phrases associated with each group. Hazard phrases can be found on chemical SDS documentation.
Dustiness. Low (Pellet- does not break up), Medium (granular or crystalline), High (fine solid or light powder/dust)
Volatility. Low, medium, high, gas. Consider boiling point of liquid and operating temperature.
Disposal e.g. autoclaving of biohazard, SU chemical disposal

PROTOCOL RISK MANAGEMENT

Secondary Containment (of protocol): e.g. open bench/fume hood/special

Open bench and fume hood for larger volumes

Measures taken to eliminate or substitute/reduce: e.g. using less hazardous, less volume of chemicals

Using less hazardous, less volume of chemicals

Personal Protective Equipment and all specific control measures Include a full description e.g. latex/nitrile/heavy gloves; safety glasses, screens; full face mask; dust mask; protective shoes; spillage tray; ear-defenders; other (state)

Emergency procedures (include first aid, fire, spillage, communication methods) N.B. full emergency plans for each chemical are detailed in individual Chemical data Sheets

Individual chemical data sheets

Is exposure monitoring required? Yes (give details) or No	**Is health surveillance required?** Yes (give details) or No
No	No

Justification and controls for any work outside normal hours (N.B. UG project students cannot work outside normal hours in a laboratory)

No outside hours

Supervision/training for worker (highlight) **N.B. All relevant training forms (e.g. for specific laboratories) should be completed**

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 ..... Alexander Bedford [redacted] .....

Name & counter-signature of supervisor..... Prof Andrew Rowley [redacted] ..... *Date*..... 01/02/2024 .....

Date of first reassessment Frequency of reassessments

5

6.3.4 Training proforma

BIOSCIENCES TRAINING PROFORMA (v1.1 AFR2023)

NAME OF TRAINEE	NAME OF TRAINER(S)	DATE(S)
Alex Bedford	Jess Minett Dr Charlotte Davies Prof Andrew Rowley	13/02/24

It is the responsibility of the PI or supervisor to determine the local training needs for each trainee and to ensure the trainee has suitable access to this training.

This form must be used to record the health and safety training **and** training in specific procedures.

This document should be updated over time (i.e. level of competency and specific procedures).

The trainer must ensure the competence of the trainee in each area before signing the form. This may be done by any or a combination of the following:

- Written test
- Oral test
- Practical demonstration by the trainee
- Reference to completed on-line training provided by University

Level of attainment competency of the trainee (use the codes A-D below and place these in the right-hand boxes within the tables)

A: The task must be directly supervised.

B: The supervisor's advice and approval must be sought before the procedure is started.

C: The work entails risks that require careful attention to safety. The trainee has been trained in the task and demonstrated competence.

D: The risks are insignificant and carry no special supervisory considerations.

BASIC LABORATORY PROCEDURES (**if field or desk-based only** then place N/A in boxes. Amend & extend table as necessary)

PROCEDURE	TRAINING ACHIEVED, METHOD OF ASSESSMENT & LEVEL OF ATTAINMENT (A-D codes)	TRAINER & DATE
Laboratory induction (add details of individual laboratories on new lines)	General induction (131a/043) - D	J Minett 13/02/24
Waste disposal	Yes - D	J Minett 13/02/24
Use of analytical balances		
Use of fume hoods		
Use of biological safety cabinets		
Use of pipettes		
Use, storage & disposal of toxic chemicals (poisons)		
Use, storage & disposal of systemic health hazards (e.g. carcinogens/mutagens)		
Use, storage & disposal of biological hazards and GMO		
Handling & disposal of sharps (e.g. needles, blades)	Disposal - D	J Minett 13/02/24

SIGNATURES:

Trainee [redacted] Date 13/02/24

Trainers/Supervisor(s)/Approvers (add as appropriate)

Name: Jess Minett Date 13/02/24

Name: [redacted] Date 13/02/24

Name: ..... Date .....

(Reassessment due.....)

COMPLETED FORM, ONCE SIGNED OFF, SHOULD BE KEPT WITH RISK ASSESSMENTS AND PROTOCOLS (i.e. in a shared TEAMS folder). IT IS THE TRAINEE'S RESPONSIBILITY TO STORE THIS FORM AND TO KEEP IT UPDATED.

6.3.5 Fieldwork risk assessment

Risk Assessment Outcome:

Risk Rating: Moderate/High risk **Submitted Date:** 25 Jan 2024
Approved Date: 1 Feb 2024 **Approved by:** Andrew Rowley

Student Details

Student Number: [redacted] **Project Supervisor:** Prof Andrew Rowley
Course: Biosciences Masters by Research Full-time **Level:** 7

6.3.5 Ethics approval

Project Title	Project ID	Owner	Date Created	Date Modified
Parasitic diseases of crabs in Swansea Bay	8411	DR Charlotte Davies	07/11/2023 03:51	08/01/2024 14:21

Showing 1 to 1 of 1 entries