Population dynamics and sex-determining mechanisms in the marine amphipod, *Echinogammarus marinus*

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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are my own and have not been submitted for any other academic award.

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Abstract

Despite their huge diversity, abundance and ecological importance, very little is still known about sex determining mechanisms within Crustacea. Sex determination in crustaceans is known to be influenced by environmental factors, via parasitic infection and genetically, however, it is possible that all three mechanisms can be involved in a single species. The gonochoristic marine amphipod *Echinogammarus marinus* (Leach, 1815) is currently being used for the development of biomarkers to measure the influence of environmental contamination on crustacean sex determination and differentiation pathways. To truly understand whether anthropogenic disruption of sex determination is currently an issue, it is critical that all the mechanisms governing the process in *E. marinus* are fully evaluated. Therefore, the aim of this project was to fill gaps in our knowledge of the general population dynamics of *E. marinus*, with a particular focus on elucidating the mechanisms of sex determination in this ubiquitous amphipod. Sex determination in *E. marinus* has been linked with feminising parasites, however, to date, no such studies have linked this species with environmental sex determination (ESD) or genetic sex determination (GSD).

This project investigated two *E. marinus* populations that differed in population structure. The Langstone Harbour *E. marinus* population (Southern England, UK) revealed no presence of parasitic sex determination (PSD). However, this study has shown that the population has a seasonal breeding pattern, with population growth and decline closely related to environmental parameters (temperature) and parasites (trematodes) respectively. The population data also revealed seasonally altered sex ratios, ranging from 36% to 71% males. ESD was recorded for the first time in an *E. marinus* population by revealing that photoperiod was the cue for sex determination. This finding was validated by a laboratory study that showed a male bias in broods that developed in long day light regimes (16h light: 8h dark) and a female bias in broods that developed in a short day light regime (8h light: 16h dark). The laboratory data and the seasonally altered sex ratios found in the field showed significant correlation with each other supporting these findings. A new species of trematode

parasite belonging to the Microphallidae family has been identified that encysts in the amphipod brain and demonstrates clear capacity for behavioural changes in its host. Individuals infected with the trematode parasite displayed distinct positive phototaxic and negative geotaxic behavioural alterations that could potentially increase susceptilbility to predation. These behavioural alterations have been linked to changes at the level of gene expression suggesting modulation of neuronal genes in the infected individuals. Putative serotonin receptor 1A, inebratied neurotransmitter, tryptophan hydroxylase and amino acid decarboxylase like genes displayed the most dramatic change in their gene expression. This represents the first study to record such changes in the neuronal pathways of parasite infected amphipods.

Another *E. marinus* population investigated from Invertkeithing (Scotland, UK) displayed a high female bias and high levels of intersexuality. The project has strengthened the evidence that PSD is present in this population with 40.4 % of the population being infected by either Paramyxea or microsporidia parasites. From the infected individuals 75% of that infection were female bias and 88.5% of intersexes, also presented an infection. The investigation explored the transmission pathways and efficiency of the parasites involved. Vertical transmission of a *Paramyxean sp*. was shown for the first time in an amphipod host and also showed the highest transmission efficiency from the mother to the eggs (96.8%). This has lead to the question of whether the microsporidian *D. duebenum* is a feminiser and has highlighted another parasite candidate for *E. marinus* sex distortion.

Despite the range of genomic techniques employed, the attempt to determine genomic sexual determination in *E. marinus* did not reveal any sex specific genomic regions. However, considering the preliminary nature of the work, this study has provided insight for future directions. Several key genes involved in sexual differentiation that presented sex exclusive expression were identified. In addition, crucial method development was performed that will allow future investigations of genetic variation in *E. marinus*. The transcriptome of the *E. marinus* has now been sequenced and along with population models enabling a greater understanding of the

links between genome and population ecology. With such a large investment in *E. marinus* as an ecological model species, it is crucial that basic biological questions and gaps in the field are addressed. Consequently, the data presented within this thesis will aid in the study of *E. marinus* and other crustaceans from the level of genetics to population effects.

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Abbreviations

°C	degrees Celsius
Acrylamide	acrylamide/bisacrylamide
APS	Ammonium Persulfate
BSA	Bovineserum albumin
cDNA	complementary-deoxyribonucleicacid
cm	centimeter
dH2O	distilled water
DNA	deoxyribonucleic acid
EDTA	Ethylenedinitrilotetraacetic acid
ESD	Environmental sex determination
EST	expressed sequence tag
ETS	external transcribed spacer
g	gravitational force
GSD	genetic sex determination
hrs	hours
kV	kilovolts
M	molar
Milliliters	ml
Minute	min
ml	milliliter
mm	millimeters
mM	millimolar
nm	nanometers
NTC	no template control
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	percentage hydrogen
PST	parasite sex determination
RNA	ribonucleic acid
RTqPCR	real time quantitative polymerase chain reaction
SDS	sodiumdodecyl sulphate
SE	standard error
SSH	selective subtractive hybridisation
TBE	trisborate ethylenediaminetetraacetic acid
TEMED	Tetramethylethylenediamine
TRIS	tris(hydroxymethyl)aminomethane
U	unit
UK	United Kingdom
V	volts
VT	vertically transmitting
µL	microliters
µL	microliters
µm	micrometers
µm	micrometers

1. General introduction

Crustacean species display a wide diversity of life history patterns and a variety of sexual strategies: parthenogenic, gonochoristic, and hemphroditic (Legrand and Legrand, 1987). In most species that sexually reproduce, the individual will differentiate during development into separate sexes. The gonochoristic marine amphipod *Echinogammarus marinus* (Leach, 1815) (see Figure 1) is currently being used for the development of biomarkers to measure the influence of environmental contamination on crustacean sex determination and differentiation pathways. However, to truly understand whether anthropogenic disruption of sex determination is currently an issue, it is critical that all the mechanisms governing the process in *E. marinus* are fully evaluated.

Sex determination in *E. marinus* appears to show a degree of plasticity, possibly being influenced by multiple factors. Sex could be determined environmentally, via parasitic infection, or genetically. However, it is possible that all three factors are involved. This project will attempt to elucidate how these factors can affect sex determination in *E. marinus* as well as population dynamics and structure of populations in the UK. The *E. marinus* transcriptome is currently being sequenced opening new possibilities to investigate molecular mechanisms involving sex determination and factors that affect population dynamics. The findings from these studies will greatly facilitate the use of *E. marinus* as a model for insight into crustacean sexual differentiation and determination and gives us a better understanding of the potential viability of *E. marinus* as a model species for monitoring environmental contamination.

1.1 *Echinogammarus marinus*

Echinogammarus marinus is an intertidal amphipod (Crustacea: Amphipoda) that is highly abundant and has a wide distribution, ranging from Polar Regions down to southern Portugal (Dick et al., 2005). *E. marinus* plays an important role in ecosystem dynamics and is a food reserve for upper trophic levels largely for bird species (Múrias et al., 1996). It is an omnivorous species that grazes on algae. They tend to reside under on sheltered beaches with muddy sediments and hard substrates (Lincoln, 1979). This species is well adapted to long emersion periods as well as estuarine conditions (Maranhao et al., 2001) and is a fairly hardy species that is tolerant to a wide range of salinities (7-34$^{0}/_{00}$) (Bettison and Davenport, 1976). As a result it is common in estuaries and other areas subject to freshwater influence (Maranhao et al., 2001). There are two phenotype colourations within the species; olive green and a less common dark red (Sexton and Spooner, 1940). In common with many amphipod species (Costa and Costa, 1999, Covi and Kneib 1995, Drave and Arias, 1995, Moore and Wong, 1996), sexual activity is partly seasonal but occurs throughout the year (Maranhao et al., 2001). Continuous reproductive output allows for no limitation when studying reproductive processes.

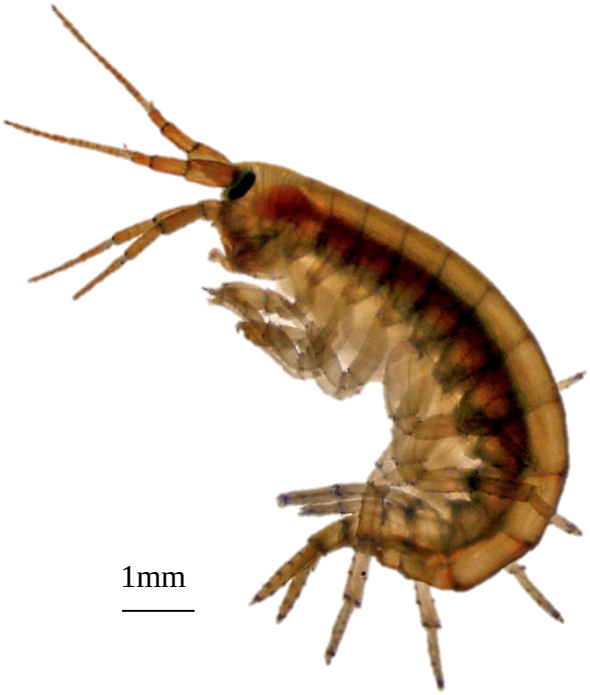

Figure 1: Female *Echinogammarus marinus* (Photo - Amaia Etxabe).

E. marinus has sexual dimorphism and males tend to grow larger than females, with females and males growing up to 20mm and 25mm, respectively (Sexton and Spooner, 1940). The gnathopods are also larger in male individuals, allowing males to compete for females and guard the females in pre-copula (Conlan, 1991). Therefore, the smaller males have reduced reproductive fitness and reduced mating success. Males possess genital papillae, while females possess brood plates (oostegites) that hold eggs within the brood chamber. Mature females also retain a pore-like structure on the carapace behind the head which has been observed to aid male attachment during pre-copula (Ford, 2004). A further characteristic that differentiates the two genders are the setae located on the uropods. The male setae can be described as hair-like, whereas female setae are spine-like structures (Sexton and Spooner, 1940).

Female fecundity strongly correlates with size in amphipods species, with larger females producing larger brood sizes (Beare and Moore, 1996, Costa and Costa, 1999, Dauvin, 1988, Ford et al., 2004, Lafrance and Ruber, 1985, Persson, 1999, Vandolah and Bird, 1980, Maranhao and Marques, 2003, Maranhao et al., 2001). However, smaller females can be more easily guarded by males and larger females can have reduced mating success as they cannot be held in pre-copula (Hatcher and Dunn, 1997). Gammarids go into a pre-copula position before egg-laying, the female moults and the eggs drop from the oviducts to be stored in the brood pouch. The male then externally fertilises the eggs from the female's brood chamber. *E. marinus* produces on average 21 eggs per brood (Cheng, 1942). These eggs are visible within the female as the female tends to be pale after the moult and the eggs have dark pigmentation during early development, becoming lighter as the embryos develop (Sheader and Chia, 1970, Ford et al., 2003). The female carries the brood to an early juvenile stage, extending their reproductive cycle beyond spawning. Reproduction, growth and moulting are interlinked and subsequent to hatching, the juveniles go through a period of growth stages shown through each moult (Sheader and Chia, 1970). After the sex is differentiated, the sexual characteristics develop after each moult. The inter-moult periods are short in early growth stages and become

progressively longer as the individual becomes mature. For female amphipods, the synchronisation of their ovarian cycle and the moulting of their chitin set exoskeleton aids in the movement of newly ovulated oocytes/embryos through the oviducts into the brood chamber (Bettison and Davenport, 1976, Borowsky, 1988).

1.2 Intersexuality

In natural and laboratory populations of amphipods a low frequency of individuals have been observed to possess both male and female characteristics (Ford and Fernandes, 2005), a condition termed intersexuality. Consequently this tends to result in reduced fecundity than that found for the true sexes (Dunn et al., 1990, Ford et al., 2003, 2004). Intersexuality has been reported across the animal kingdom (Reinboth, 1975), and is found in a wide range of crustaceans (Dunn et al., 1990, Jungmann et al., 2004, Olmstead and LeBlanc, 2007, Sagi et al., 2002, Ford, 2012).

The term intersexuality or intersex has often been confused within the literature with other sex phenotype conditions such as hermaphroditism and gynandromorphism (Narita et al., 2010). Compared with plants and some animal groups, hermaphrodites encompass male and female forms as their normal life history and are extremely rare within crustaceans (Narita et al., 2010). In contrast, intersexuality is thought to be a consequence of some form of disruption in the sex differential pathway (Ford et al. 2003). Another term often used interchangeably with intersex is gynandromorphy and are described as sexual mosaics in which male and female tissue occurs on the same individual (Olmstead and Leblanc, 2007). These genetic chimeric individuals occur when genes that govern sex determination/differentiation pathways are altered during the development of the zygote resulting in some regions forming as one gender and the other areas forming as the other (Narita et al., 2010). Once the gynandromorph individual develops the male and female tissue can be seen in clear borders distributed bilaterally, patchily or uniformly mixed. Therefore, a gynandromorph is a genetically chimeric individual whilst intersexes are genetically uniform (functioning as a singular sex) (Narita et al., 2010). There is no evidence, to our knowledge that *E. marinus* or other gammarids are hermaphrodites or contain gynandromorphs within their populations. Within this study, intersex is termed as an individual that possesses characteristics from both genders, but reproductively functions as one gender.

Intersexes are associated with the endocrine regulated sex differentiation process in crustaceans (Sagi and Khalaila, 2001) and is believed to occur due to multiple factors; disruption of the androgenic gland (AG) (Charniaux-Cotton, 1958), sex distorting parasites (Rodgers-Gray et al., 2004), environmental sex determination (ESD) (Dunn et al., 1996) and possibly chemical/anthropogenic pollution (Ford et al., 2006, Olmstead and LeBlanc, 2007, Short et al., 2012b).

Intersex *E. marinus* individuals tend to be of larger size than normal sex phenotypes (Ford et al., 2003). Intersex males can be either external intersex, in appearance look male and possess rudimentary brood plates, or be internal intersex, possessing gonadal abnormalities such as, the development of oviduct like structures (See Figure 2b; Ford et al., 2008). External intersex males can sometimes possess an oviduct structure internally, although internal intersex males never display external characteristics such as brood plates. Intersex females in appearance look female, however, they possess one or two genital papillae (See Figure 2a). It is unclear whether different phenotypes of intersexuality occur via different mechanisms or whether it is the same mechanism acting with varying degrees of severity to cause the multiple variations of intersexuality (Ford et al., 2003, Short et al., 2012b).

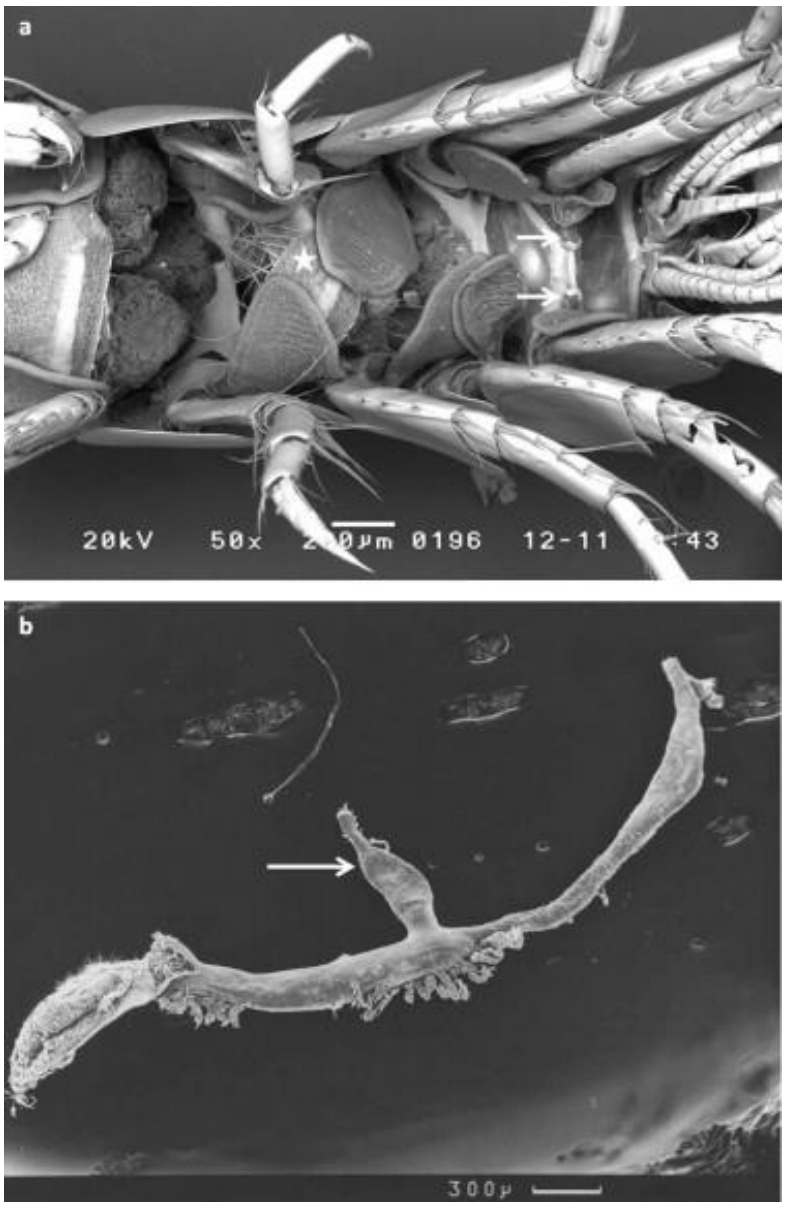

Figure 2: Intersexuality in amphipods (a) externally intersex *Gammarus minus* displaying brood plates (star) and genital papillae (arrows) and (b) internally intersex *Echinogammarus marinus* displaying testes with an oviduct (arrow) taken from Ford et al., (2008).

There are a number of reported costs associated with the intersex phenotype. It is suggested that intersexes mature at a larger size due to energy shift towards somatic growth rather than reproductive development (Ford et al., 2004). Large size along side with morphological abnormalities and possible pheromone dysfunction can severely reduce pairing success (Ford et al., 2004, Kelly et al., 2004). Reduced fecundity, fertility, embryo survival and delayed maturation have been documented with *E. marinus* intersexes (Ford et al., 2003, 2004). Intersex *E. marinus* females produce ~20% less eggs than normal females and 10% fewer eggs from intersex females produce fully developed embryos compared to normal females (Ford et al., 2004). Yang et al., (2008) revealed that intersex males have reduced sperm counts (~15%) compared with normal individuals and that *E. marinus* populations situated in polluted sites had 30% more intersexes. Ford et al., (2006) showed significantly higher prevalence of intersexuality in the polluted sites compared with reference sites and that the intersexes were more likely to be infected with microsporidia. However the lack of microsporidian infection within the intersex population at the reference site indicated that parasitism is not the sole cause of intersexuality within the populations studied (Ford et al., 2006, Yang et al., 2011, Short et al., 2012b). A direct effect has not been proven however there is a strong relationship with pollution and intersexuality. The reasoning for this could be increased host susceptibility when under polluted conditions.

Using simulations of varying sex ratios, fecundity, intersex levels and mortality rates it was concluded that intersexes can have dramatic effects on populations (Ford et al., 2007). This work was theoretical however recent work by Martins et al. (2009) has been performed using field data from populations of *E. marinus*. This study demonstrated intersexuality incidence, sex ratio fluctuations and intersex reproductive output. Establishing how these factors affect population dynamics of a species gives us a better understanding of the consequences of intersexes have within natural populations (Martins et al., 2009). The research revealed that female biased populations were less sensitive to intersexes and reduction in reproductive output. Yet there are thresholds and, if sex ratios become too unbalanced the population can face extinction or collapse (Hatcher et al., 1999). Martins et al. (2009) demonstrated through modelling that the impact that intersexes have upon a population are

contributed to many factors such as sex ratio fluctuations, intersex reproductive output and intersexuality incidence and highlights how intersexuality can affect *E. marinus* population dyanmics.

The causes and mechanisms behind intersexuality are not fully understood. Past studies have shown that costs are high with intersexuality with individuals having reduced reproductive output compared with normal individuals. In spite of this, intersexes are present and persist within natural populations (Ford et al., 2008). Despite past work, such as the sex allocation theory, that suggests intersexes would be selected out due to their lack of reproductive fitness (Charnov, 1982). Their persistence in population implies that there are a mixture of processes and factors that are involved with intersexuality and in the broader scale sex determination that are not fully understood. A better knowledge of sex determination and differentiation especially in genetic mechanisms would greatly facilitate the study of intersexuality. This may provide some insight into the causes of intersexuality, the genetic manipulation needed to become an intersex individual, as well as why *E. marinus* appears to have various phenotypes of intersexuality.

1.3 Sex determination in Crustacea

Sex determination in animals is diverse and can vary dramatically over short phylogenetic distances (Bull, 1983). The archaic group, Crustacea, comprises of a large portion of the arthropods and consists of approximately 50,000 species that inhabit the majority of ecological niches. This ecologically relevant group can demonstrate high sexual plasticity making the study of sex determination somewhat problematic in some species. There are multiple factors believed to trigger or influence sex determination in Crustacea and sex can be determined by environmental (Bulnheim, 1978), parasitic (Mautner et al., 2007), as well as genetic factors (Legrand & Legrand, 1987).

Sex determining mechanisms drive a population's sex ratio, which in turn affects the size of the reproducing population. Fisher's principle of equal investment states that natural selection favours equal frequency of males and females (Fisher, 1958). This evolutionary theory of a stable 1:1 sex ratio model has been generally favoured when producing males or females has similar costs (Fisher, 1958, MacArthur, 1965, May, 1983). In crustaceans, however, it is rare to find a species with an unbiased sex ratio (Saher and Qureshi, 2011, Prato et al., 2009, Doi et al., 2008, Castiglioni and Buckup, 2008, Litulo, 2005, Maly, 1970, Lasker et al., 1970, Ford and Glazier, 2008). This can be a consequence of a gender bias in the production of offspring, or mortality rates could be sexually differentiated, such as, cases of sex biased predation (Appadoo and Myers, 2004). Molecular mechanisms of sex determination in crustaceans are still largely unknown with the only well characterised arthropod sex determination pathway being that of the highly divergent insect, *Drosophila melanogaster* (Sanchez & Lucus, 2008).

In the fruit fly *Drosophilia*, each cell determines its sex independently at a very early embryonic stage and continues during later development through a gene cascade comprising of *Sex-lethal (Sxl)*, *transformer (tra)*, *doublesex (dsx)* and several other regulatory genes, in which differential splicing of mRNAs of these genes perform a

crucial role in (see Figure 3) (Nothiger and Steinmannzwicky, 1985, Schutt and Nothiger, 2000). Sex determining mechanisms in other arthropods are still unclear, in insects it is suggested that that there is a single model consisting of a master regulatory gene at the top of the cascade and a highly conserved *(dsx)* gene at the bottom (Narita et al., 2010). Kato et al. (2011) recently revealed the role played by the highly conserved *Doublesex* gene in the parthenogenic crustacean, *Daphnia magna*. Instead of sex being regulated at the level of pre-mRNA splicing in the coding region, the *Daphnia Dsx* gene sexually differentiates through the transcript number. Increased expression was found only in male individuals during embryogenesis and the knock-down of the *Daphnia Dsx* gene in male embryos lead to the production of female traits (Kato et al., 2011). This study has shown that the *Daphnia Dsx* gene is a crucial component of regulating the male phenotype and has linked a genetic element with a species that utilises ESD.

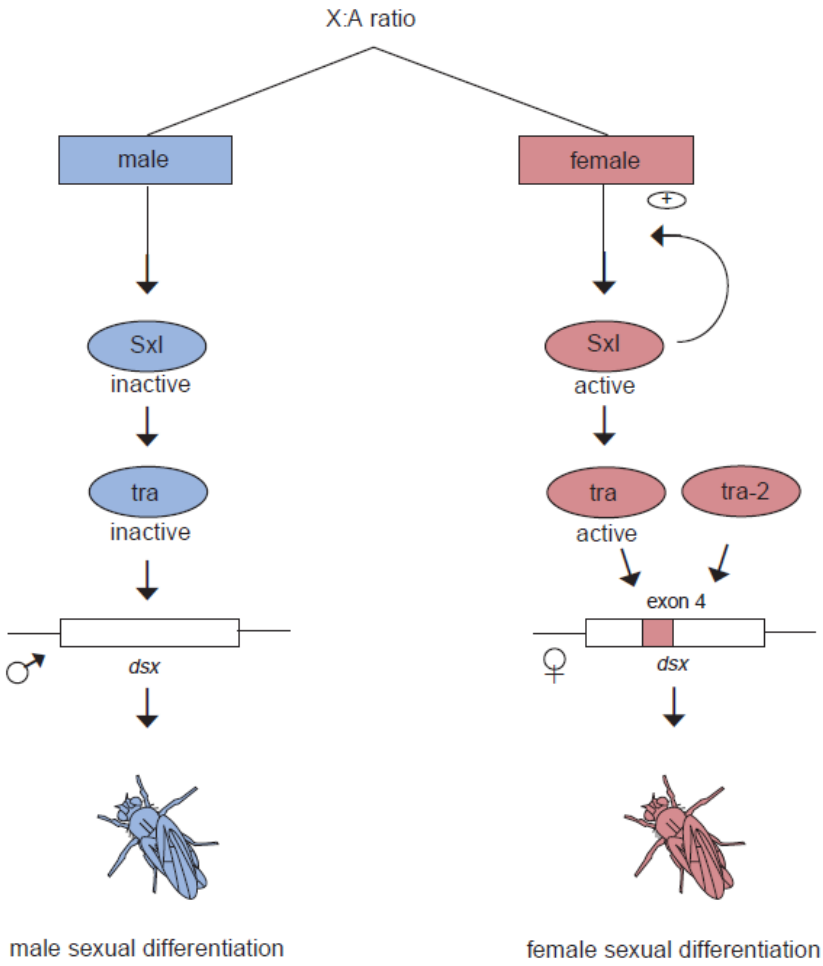

Figure 3: Sex determination pathway in Drosophilia that consists of a splicing cascade, in which each pathway regulates the other. The male default pathway yields male offspring with the splicing of sex lethal (sxl) being non functional. The female splicing pathway of sex lethal (sxl) produces the x chromosome/autosome ratio (1:1) and initiates positive feedback loop by inhibiting the male default splicing pathway, similarly to the transformer (tra) gene. The proteins tra and tra-2 control the insertion of exon 4 to produce the doublesex (dsx) variant that denotes the female phenotype. Splicing out exon 4 in the default pathway produces the male phenotype. Taken from Herbert and Rich (1999).

1.3.1 Sex chromosomes

In the majority of arthropods, sex is genetically determined. Chromosomes that determine an individual's gender are often termed 'sex chromosomes', in which one of the two chromosomes are commonly degenerate (Bull, 1983, Charlesworth and Charlesworth, 2000). The X and Y (or Z and W) are morphologically distinguishable with high amounts of repetitive DNA (Bull, 1983). Within the XY system, females are the homogametic sex (XX) and males are the heterogametic sex (XY). In the ZW system, males are the homogametic sex (ZZ), while females are heterogametic sex (ZW) (Legrand et al., 1987). Genetic sex determination in Crustacea is diverse among species with male heterogamety being present in Amphipoda, Decapoda and Ostracoda, and female heterogamety in Branchiopoda (Legrand et al., 1987). Heterogamety is exhibited in both males and females in Copepoda and Isopoda (Lecher et al., 1995). However, the absence of cytological detection of sex chromosomes within studies does not signify that heterogametic sex determination is absent (Legrand et al., 1987). This lack of knowledge has created difficulties in understanding sex determining mechanisms and its evolution within crustaceans.

Cytogenetic parameters such as chromosome number and structure aid in taxonomy and to identify phylogenetic relationships as well as giving an insight into the genetic structure of the species or population in question (Thiriot-quievreux and Cuzinroudy, 1995). Karyological studies in Crustacea have been problematic due to lack of methodologies for adequate preparations to produce high quality metaphase plates. The main obstacle preventing accurate counting of crustacean chromosomes is the high diploid numbers, small chromosome size and generally the small size of the species making retrieving tissue difficult (Coleman, 1994). However, despite this reliable karyotype research has been obtained in many crustacean groups such as Euphausiacea (Thiriotquievreux and Cuzinroudy, 1995), Copepoda (Lazzaretto et al., 1989, Standiford, 1989), Decapoda (Deiana et al., 1996) and Isopoda (Dicastro et al., 1989, Dicastro et al., 1977, Dicastro et al., 1979).

Chromosome number has been found in 90 amphipod species and has been proved to be highly variability (Coleman, 1994). Chromosome size varies from 2 to 5 µm and diploid number from 18 to 68 (Lecher et al., 1995). However 26 are considered as the modal haploid number found in many of the Gammaridae (see Figure 4). *E. marinus* displayed chromosomal polymorphism with haploid chromosome numbers of 25 and 26 (Orian, 1957). There are several possible explanations for this karyological difference; increase or decrease of chromosome number during evolution via fission or fusion, extreme increase in chromosome number due to polyploidy (Salemaa, 1984), and restricted increase due to supernumerary chromosomes (B-chromosomes). However, polyploidy has been ruled out in gammarids evolution (Orian, 1957). Supernumerary chromosomes are add-ons to the chromosome set that have no apparent positive or negative effect for the species. This generates intra specific variation which sometimes can be geographically restricted (Orian, 1957).

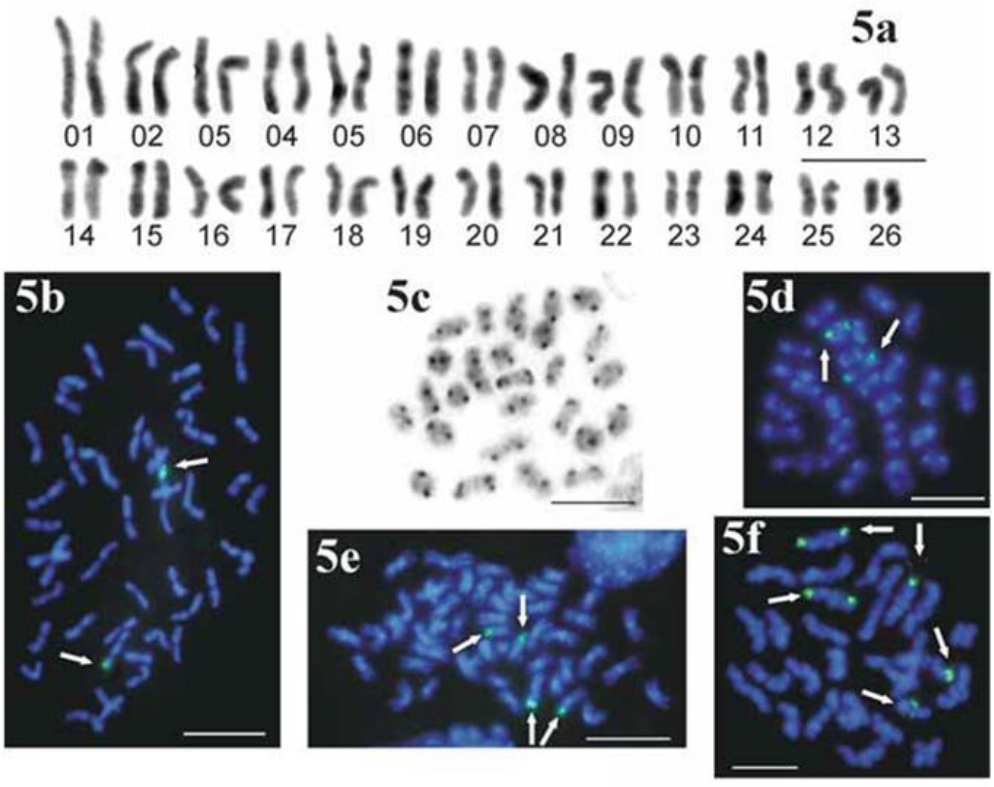

Figure 4: Taken from Libertini and Rampin (2009), an extensive study into Gammaridae karyotyping. 5a. *Echinogammarus obtusatus* 5b. *E. obtusatus* embryo (45S rDNA FISH) 5c. *Echinogammarus finmarchicus* spermatocyte (C-banding) 5d. *E. finmarchicus* spermatocyte (45S rDNA FISH) 5e. *E. finmarchicus* spermatogonial (45S rDNA FISH) 5f. *Gammarus oceanicus* spermatocyte (45S rDNA FISH).

The most reliable methodology to produce good plates for karyotyping is aceto-carmine-staining to create squash preparations of the testes. This can also be done with squash preparations of oocytes prior to oviposition. Ovaries are fragile, adhesive to each other and difficult to free from wall tissue. The most informative stage of the eggs and testes for karyological study is the first meiotic metaphase. Unfortunately, preparations are technically difficult as this stage is short in duration (Orian, 1957). An alternative technique for obtaining mitotic chromosome metaphase in marine amphipod species has been using embryos (fertilised eggs) for sample tissue (Campos-Ramos, 1997). Although, this technique could not be adopted for identifying sex chromosomes as the gender of embryos would not be known.

Despite the volume of crustacean karyological studies, the knowledge is still limited due to technical details discussed. Sex chromosomes are not well differentiated within amphipods, with the exception of a single account of *Ansiogammarus annandalei*, in which it was noted that males obtain heteromorphic bivalent (XY-XX type) which condense during early division I (Niiyama, 1950). However, no other karylogical studies have validated these findings or shown evidence of sex chromosomes in other amphipod species, to our knowledge. Due to the technical difficulties in obtaining good quality metaphase plates and the presence of B-chromosomes contributing to the uncertainty of sex chromosome identification, other avenues have been explored to identify genetic sex determination in crustacean species. Female heterogamety can be demonstrated by crossing two genetic females with one being experimentally sexually reversed to ensue as a functioning neo-male if such crosses produce all female viable broods, it indicates polychromatism.

Suzuki (1999) ran cross breeding experiments using the ispod *A. vulgare*. Normal males vs. androgenic gland (AG) ablated males (neofemales) and normal females vs. AG implanted females (neomales) demonstrated influential effects on sex ratios of the broods, proving that to a certain extent, genetic or chromosomal sex determination mechanisms are in place within this species (Suzuki, 1999). Parnes et al. (2003) conducted cross breeding with the Australian red-claw crayfish, *Cherax quadricarinatus* in which they cross bred varying combinations of intersex

individuals (See Figure 5). This showed that the control group of normal males and females yielded 1:1, whereas crosses of females with an intersex male produced 1:3 (male:female) sex ratio, demonstrating that intersex functioning males are genetically female (WZ) and that the male is the homogametic (ZZ) sex. The study further went on to breed normal males with females from the progeny of the intersex father. This yielded nearly 100% females supporting the initial finding of the suggested sex determination model (Parnes et al., 2003). To date, cross breeding experiments have been the best technique for understanding genetic sex determination within Crustacea.

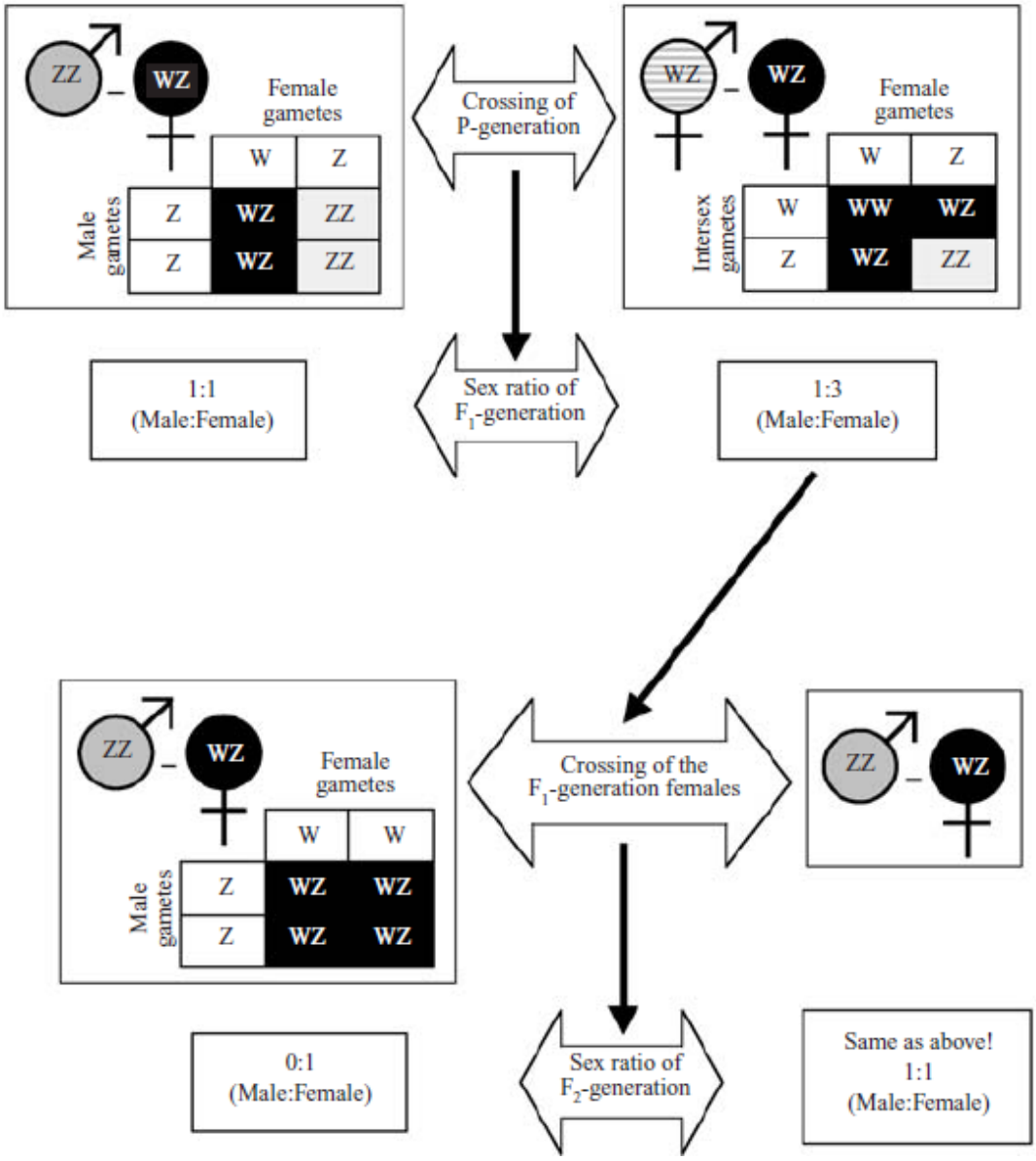

Figure 5: Sex heritability model of *Cherax quadricarinatus* taken from Parnes et al. (2003).

1.3.2 Sex hormones

In the past, the existence of sex hormones within insects has been debated (De Loof and Huybrechts, 1998). Insect sex determination is believed to be a strictly genetic process without the use of hormones (Maas and Dorn, 2005). In crustaceans the fundamental mechanisms that regulate sex differentiation are through the male androgenic gland (AG). Charniaux-Cotton (1954) first discovered that sex determination in Crustacea was under hormonal control. The source responsible for male determination and the inhibition of female differentiation is the hormones secreted by the AG (Charniaux-Cotton, 1954). Without the presence of the AG individuals are female by default. The AG synthesises and secretes the AG hormone which can control primary (spermatogenesis) and secondary (external morphology) sex characteristics (Nagamine et al., 1980a). Manipulation of the AG hormone by either removal or implantation of the AG has shown to affect many physiological and morphological processes which seem to vary among species (Sagi et al., 1990). In the majority of Crustacea studies, the AG is close to the sub terminal region of the vas deferens between the muscles of the last thoracic leg within the coxopodite (Charniaux-Cotton, 1958, Charniaux-Cotton, 1960). The AG cells have common characteristics across different species; well developed granular endoplasmic reticulum and golgi apparatus, mitochondria with flat and transverse cristae and numerous lysosomes (Hasegawa et al., 1991).

In the amphipod, *Orchestia gammarellus* implantation of the testis or genital tract without the AG caused no effect within the female. The implantation of the AG into a juvenile female caused the female to revert to a male, while implantation in a mature female caused masculinisation of primary and secondary sex characteristics (Charniaux-Cotton, 1954). After implantation, the first consequence observed was inhibition of yolk formation. Subsequently, the female's appendages developed progressively during intermolt period's equivalent to a normal male. Ovary gonia formed secondary gonia that split into functional testis producing spermatozoa, spermatids and spermatocytes (Charniaux-Cotton, 1962). In addition the removal of the AG from a male caused the male to become sexually undifferentiated. For example, removal of the gnathopod caused the regenerated limb to be characterised

neither male nor female. Within the gonads spermatogenesis diminished. If an ovary was implanted into a normal male the ovary would typically be transformed into a testis. However with individuals that had the AG removed the ovary was maintained within the male unaffected. (Charniaux-Cotton, 1962). The research by Charniaux-Cotton demonstrated that the AG was the lone source of the hormone that was responsible for male characteristics and development.

The AG has been manipulated in many species mainly isopods (Suzuki, 1999), amphipods (Charniaux-Cotton, 1954) and decapods (Malecha et al., 1992). These physiological manipulations have generally had higher impact on juveniles and success rates have correlated with the subjects age (Sagi and Khalaila, 2001). The prawn, *Macrobrachium rosenbergii* has displayed complete sex reversal by removal or implantation of the AG in early juvenile stages and had the ability to mate with normal individuals and produce offspring. (Malecha et al., 1992, Nagamine et al., 1980ab, Sagi et al., 1990).

Purification, identification, DNA sequencing and cloning of the gene encoding the AG hormone of *A. vulgare* has been accomplished (Okuno et al., 1999, Okuno et al., 1997, Martin and Juchault, 1999, Martin et al., 1999, Nagasawa et al., 1995). The structure of the AG hormone of *A. vulgare* has been revealed to be unstable thermodynamically and is considerably less favourable as more energy is required for synthesis and maintaining its form. The basis for this less stable form is thought to be intended for faster degradation giving a greater control of the proteolytic pathway which in turn allows the organism to have strict control of sex determination (Katayama et al., 2010). The hormone shows similarity to the pro-insulin superfamily of peptides (Martin et al., 1999). Another two species of isopods, *Porcellio scaber* and *Porcellio dilatatus* have had their AG hormone identified and sequenced. It appears that the AG hormone is highly conserved among the three isopod species (Ohira et al., 2003).

Hormones from the insulin family are generally not linked as gender specific, however, increasing evidence is suggesting a possible association of insulin pathways in sexual differentiation (Manor et al., 2007, Manor et al., 2009, Ventura et al., 2009a, Ventura et al., 2009b, Nef et al., 2003). Manor et al. (2007) first constructed a decapod AG cDNA subtractive library revealing insulin like gene expressed exclusively in the AG of male *C. quadricarinatus*. However its resemblance to the three isopod AG hormones previously sequenced was low (between 16 and 19% identity) (Ohira et al., 2003, Okuno et al., 1999). Recently silencing of an insulin-like gene expressed specifically in the AG library of *M. rosenbergii* (*Mr*-IAG) was the first functional sex reversal via this method (Ventura et al., 2009b). The neo-females produced all male progeny demonstrating that manipulating this key sex determining gene can be extremely useful for further GSD studies (Ventura et al., 2012). These studies support the concept that insulin may have evolved from a sex differentiation background within isopods and decapods. However no amphipod AG hormone to date has been identified. From examining studies of the crustacean AG we can conclude that it can exert distinct effects within the animal's morphology, physiology and behaviour. The pivotal role of the AG in sex determination has been demonstrated by implantation and ablation experiments within many crustacean species. However, it still remains unknown on which pathways are influenced by the AG and how the hormone controls sex differentiation mechanisms.

1.3.3 Sex distorting parasites

Parasitism is a highly influential factor in the reproductive output of a population (Forbes, 1993). Parasites can be categorised by their mode of transmission; vertical (from infected parent to offspring) or horizontal (through contact from either an infected individual or a free living parasitic stage). Horizontally transmitted parasites are generally pathogenic and tend to decrease the reproductive success of the host due to host resources being reallocated to the parasite. Vertically transmitted parasites are associated with low pathogenicity due to host and parasite fitness being closely linked (Dunn and Smith, 2001). Within crustacean species, extreme female biased populations have been linked with parasitic influences (Terry et al., 2004). These sex biased ratios have been associated with either infestations of transovarially transferred parasites, which have a feminising effect on host offspring, or with male killing (Kageyama and Traut, 2004, Ironside et al., 2003). This sex ratio distortion gives for a highly effective evolved strategy as a male host is as a dead end due to their modes of transmission thereby female broods are favoured as the transmitting gender (see Figure 6) (Bandi et al., 2001).

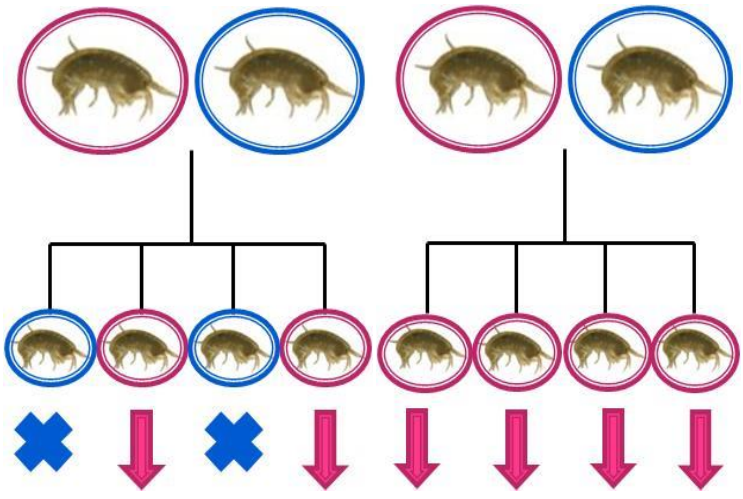

Figure 6: A strategy in which feminising the brood increases transmission, a male host is seen as a "dead-end", thereby female broods are favoured as the transmitting gender.

Rather than feminisation of host broods, *Wolbachia* in the moth species, *Ostrinia scapulalis* induces sex distortion by male killing (Kageyama and Traut, 2004). This was discovered when exploring the presence or absence of sex chromatin in larvae at the hatching stage. Unhatched larvae displayed significant biased towards genetic males producing overall females only. Male killing is thought to be due to the intolerance of feminisation within the host, which is related to genetic backgrounds or differences (Kageyama and Traut, 2004). This allows the reallocation of resources back to females' thus increasing transmission. Male killing mechanisms are widespread within insect species with no known cases of male killing mechanisms within amphipods. However, male killing should not be ruled as a possible mechanism when looking at sex distorting parasites.

The knowledge of the mechanisms that result in parasitic feminisation and intersexuality in amphipods is only starting to be established. Rodgers-Gray et al. (2004) was one of the first studies to link parasite induced intersexuality and feminisation in amphipods with the inhibition of AG development and hormone production. Following on from this study, Ford et al. (2005) looked at the four different sexual phenotypes within *E. marinus* (normal and intersex male, normal and intersex female) to compare androgenic gland (AG) activity quantified via MALDI-TOFF spectrometry. It showed reduced androgenic gland activity in the infected intersex male compared with the normal male indicating that intersex is a result of disturbance within the androgenic gland (Ford et al., 2005).

Host genes that are targeted by *Wolbachia* are different between insects and crustaceans. For example, within insects the master regulator genes that influence somatic sex determination (sex lethal and double sex genes) are hypothesised to interact with the bacteria. This is thought to be due to the lack of sex hormones in insects (Negri et al., 2006). Whereas in crustaceans it has been proposed that *Wolbachia* feminise isopods in a similar approach to microsporidia with AG disruption (Bouchon et al., 2008) .

It is thought that, with the exception of determination of offspring sex, vertically transmitting parasites have little or no detrimental effect on the host (Ironside et al., 2003). This would fit the prediction that vertically transmitting parasites will only inflict either a positive or neutral effects on the host as parasite and host fitness are entwined. Although it has been reported in a few cases that transovarially transmitted microsporidian parasites can cause reductions in egg production, hatching (Andreadis and Hall, 1979) and survival (Raina et al., 1995). Haine et al. (2004) highlighted a positive effect on host reproduction in two species of microsporidia (*Nosema granulosis and Dictyocoela muelleri*) infecting the amphipod *Gammarus roeseli*. Infected females were observed in the field and laboratory to breed earlier in the reproductive season compared to the uninfected females, giving earlier host reproduction and thus increasing number of host broods (Haine et al., 2004). Later breeding experiments with infected *G. duebeni* with *N. granulosis* found that brood survival increased compared with broods from uninfected mothers (Haine et al., 2007). Conversely, another study looking into sperm allocation revealed that *G. duebeni* infected with microsporidia would receive less sperm from a male than an uninfected female (Dunn et al., 2006a). This leaves the hypothesis that vertically transmitting parasites either maintain or improve host fitness open to question.

1.3.4 Environmental sex determination

Environmental sex determination (ESD) occurs when sex is established through non-genetic cues (abiotic factors) through a period after egg fertilisation (Korpelainen, 1990). ESD is widespread and has been documented in diverse groups of organisms including Echiura, reptiles, fish, nematodes and crustaceans (Adams et al., 1987, Korpelainen, 1990, Ciofi and Swingland, 1995, Conover and Kynard, 1981, Bull, 1980, Petersen, 1972). Sex determination can be influenced by a variety of cues such as temperature, day length, salinity, pH, population density and nutrient availability (Barón et al., 2002, Dunn et al., 2005, Voordouw and Anholt, 2002, Zupo, 2000). ESD generally occurs in early development where the epigenetic factors influence gene expression within the zygote. However, in some cases the epigenetic factors fix the sex by acting on gametogenesis of a female (Bulnheim, 1978, Bull, 1983). Not all populations within a species necessarily possess ESD and it is more prevalent in populations that have a limited breeding season (Watt and Adams, 1994). This variation in ESD indicates the adaptive response of reproductive strategies under varying environmental conditions, allowing an individual to develop into the gender that provides the best ecological fitness at the time, given the environment they encounter (Naylor et al., 1988b, Watt and Adams, 1994). It has been suggested that selective forces drove the transition from GSD to ESD in populations (Bull, 1981) and temperature sensitive mutations artificially produced in *Drosophila melanogaster* and *Caenorhabditis elegans* have demonstrated how GSD has the capacity to rapidly evolve into ESD resulting from a control gene mutation (Epper and Bryant, 1983, Hodgkin, 2002). The ability to skew sex ratios via environmental parameters promotes reproductive fitness and has ensured the evolution of ESD mechanisms in many reproductive systems.

A well documented case of ESD in vertebrate species is temperature sex determination (TSD), one of the most prominent types of ESD, it is found in reptiles, including all crocodilians, some lizards, and many turtle species (Bull, 1980). The sex of individuals is permanently determined by thermal conditions during egg incubation in the middle trimester of their embryonic development (Janzen and

Paukis, 1991). The red eared slider turtle produces female egg clutches at 31^{o}C and male egg clutches at 26 ^{o}C, the intermediate temperature (29.2 ^{o}C) produces a 50:50 sex ratio (Weibbels et al., 1991). The small temperature range between all male and all female clutches means that local temperature shift and global warming can drastically skew population sex ratios having great ecological impact.

The crustacean *Daphnia magna* is known to switch from parthenogenic to sexual reproduction when environemental quality declines (Hebert, 1978). Broods of female offspring are increased under favourable conditions. The daphnid population then increases through asexual reproduction. When environmental cues occur, such as reduced light period and reduction of diet, the population produces male biased offspring and undergoes sexual reproduction (Hebert, 1978). *D. magna* has been observed to have significantly different sex ratios in four different geographical locations in the same breeding season revealing how varied sex ratios can be within populations that possess ESD (Barker and Hebert, 1986).

Diet has been seen to influence sex ratios in copepods and shrimp (Irigoien et al., 1999, Zupo, 2000, Zupo and Messina, 2007). The protandric shrimp, *Hippolyte inermis* has been observed to reverse sex when influenced by a diatom diet. Through histological study it was seen that the diatom species, *Cocconeis* caused disruption of the testes and AG, which lead to the production of an ovary and subsequent development of *beta* females. This has been observed in the laboratory and the field (Zupo, 2001). The disruption of the male gonads was suggested to be due to apoptosis (Zupo and Messina, 2007) occurring during the post larvae stage in early sex maturation (Zupo, 2000). However the mechanisms that trigger this programmed cell death are unknown.

G. duebeni is a well documented example of ESD within amphipods with it being shown that photoperiod and temperature influences sex ratios (Naylor and Adams, 1987, Naylor et al. 1988ab, Watt and Adams, 1993, 1994, Dunn et al. 1996, 2005) (See chapter 4 for further details). Although descriptions of ESD mechanism in other

species within this crustacean group are somewhat lacking. Further investigation is required to determine whether environmental cues can influence sex determination in a variety of amphipods or if *G. duebeni* is an isolated case. This could possible answer interesting evolutionary questions regarding sex determining mechanisms in Crustacea and gain a better understanding of how the environment can influence population dynamics and the physiology of amphipod species.

1.4 Aims and objectives

The majority of the literature describing *E. marinus* is aimed at developing a better understanding of intersexuality and the factors that induce it, as well as ecotoxicology studies that focus on how anthropogenic pollution can affect gammarids. The aim of the studies described within this thesis is to fill gaps in our knowledge of the general population dynamics of *E. marinus*, with a particular focus of attempting to elucidate the mechanisms of sex determination in this ubiquitous marine amphipod. Sex determination in *E. marinus*, has been linked with feminising parasites. To date, however, no such studies have linked this species with environmental sex determination (ESD) or genetic sex determination (GSD).

Specific objectives to be addressed in this thesis include:

- To determine the population dynamics of a natural population of *E. marinus* from Langstone Harbour (Portsmouth, UK) and establish the population sex ratios and seasonality of breeding.
- To establish parasite groups infecting the Langstone Harbour *E. marinus* population and then determine temporal changes in these parasite groups, some of which are associated with sex ratio distortion or a potential of great influence over host population abundance.
- To determine whether *E. marinus* possesses environmental sex determination (ESD) through laboratory study and whether this links with sex ratios observed in the field.
- To determine the role of feminising parasites in sex determination by studying an *E. marinus* population presenting high female bias.
- Use various techniques to establish whether heterogametic sex determination is present and attempt to identify sex specific genomic regions that could act as a genomic sex marker for *E. marinus*.

2. The population dynamics of *Echinogammarus marinus* at Langstone harbour

2.1 Introduction

The biology and ecology of *Echinogammarus marinus* have been studied at various latitudinal locations (Maranhao et al., 2001, Maranhao and Marques, 2003, Skadsheim, 1982, Skadsheim, 1984, Van Maren, 1975a, Van Maren, 1975b, Vlasblom, 1969, Pinkster and Broodbakker, 1980). General observations from the *E. marinus* populations studied shows seasonal change in *E. marinus* density, with peaks in spring and summer months and continuous recruitment throughout the year. Some *E. marinus* populations do display a univoltine life cycle in cold temperate to sub-polar regions (Denmark), in which they breed during April to June (Skadsheim, 1982) and other populations found in maritime temperate climates (Normandy, France) display a multivoltine breeding season from May to June (Pinkster and Broodbakker, 1980).

The study of parasites is now a shared common interest of both ecologists and parasitologists due to their pivotal role in community structure and ecosystem dynamics (Lefevre et al., 2009, Poulin and Mouritsen, 2006). Parasites are highly abundant organisms and can account for a substantial portion of total biomass in ecosystems (Kuris et al., 2008). However their functional importance in terms of population dynamics and ecology has only become realised in recent years (Holmes, 1996, Horwitz and Wilcox, 2005, Hudson et al., 2006, Lefevre et al., 2009, Wood et al., 2007). Therefore, population studies that focus on abundance of a species should include the prevalence of certain parasites as a determining factor. It has been shown that trematodes infecting gammarids can affect mate choice (Thomas et al. 2005). Acanthocephala and trematoda parasites can affect the location of amphipods in the water coloumn and other swimming behaviour altering their chances of being

predated (Bethel and Holmes, 1977, Thomas et al. 1995), sex distorting parasites, such as microsporidia (Dunn and Hatcher, 1997b, Terry et al.,2004), *Wolbachia* (Kondo et al. 2005) and Paramarteilia (Ginsberg-Vogel, 1991) can dramatically affect population structure, in particular sex ratios and levels of intersexuality (Terry et al., 2004). For these reasons these parasite groups will be screened to see whether they are present within the *E. marinus* population at Langstone Harbour.

Extensive population studies have been conducted on *E. marinus*, mainly at the northern and southern latitudes (Maranhao et al., 2001, Maranhao and Marques, 2003, Skadsheim, 1982, Skadsheim, 1984, Van Maren, 1975a, Van Maren, 1975b, Vlasblom, 1969, Pinkster and Broodbakker, 1980). This study will gain a better understanding of the population dynamics within the *E. marinus* Langstone Harbour (England, UK) population, a mid-latitude population. This was achieved by observing seasonal fluctuations in abundance of *E. marinus*, collecting length/frequency data to determine size at maturity and establishing the population's breeding seasons. In addition, a number of key parasite groups to have the potential to influence population structure were determined and their seasonal prevalence established. Field data collected within this chapter will provide baseline information on the test population for other chapters within this thesis.

2.2 Materials and Methods

2.2.1 Population Study

To assess the population dynamics a field study was undertaken over a two year period. *E. marinus* were collected between December 2009 to December 2011 from Langstone Harbour (50º47'23.13N 1º02'37.25W) situated in Portsmouth, UK (See Figure 7). Samples were taken during low tide by selecting five $1m^2$ quadrats (total area = $5m^2$) in the intertidal zone during low tide. All algae and surface sediment (approximately 2cm in depth) was retrieved and stored in polythene bags. In the laboratory, samples were washed and decanted through a 0.7 mm sieve and all algae were scraped to ensure no individuals were left.

All amphipods were collected and stored in 70% ethanol and *E. marinus* specimens were separated into males, females and juveniles. Generally sex could be determined within individuals that were approximately over 10mm in length. *E. marinus* males were distinguished by the presence of enlarged gnathopods and genital papillae. Females were distinguished by much smaller gnathopods and oostegites (brood plates) (see Figure 8). Individuals not presenting any of these features were grouped as juveniles. External intersex specimens were also recorded. To record seasonal reproductive levels any females carrying embryos or juveniles in their brood pouch were also noted. Lengths were measured from anterior end of head to distal end of telson (see Figure 9) via photomicrographs (Leica 560, JBCKL-F1030U and UTHSCSA Image tool).

Figure 7: Illustration of the sample site for the collection of *Echinogammarus marinus*. (A) shows Langstone harbour with (B) illustrating the precise sample site (50º47’23.13N 1º02’37.25W), (C) photograph of sample site appearance. Image (A,B) was produced by Google Earth Software (2011).

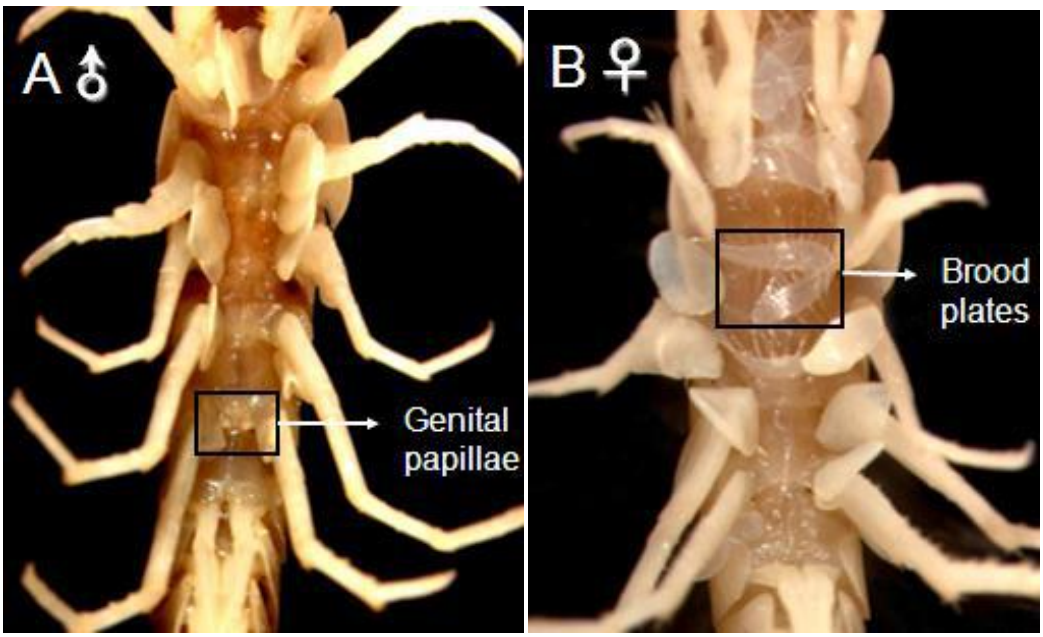

Figure 8: *Echinogammarus marinus* males and females are distinguished by the presence of genital papillae (A) and brood plates (B), respectively.

Figure 9: Photomicrograph of a male *Echinogammarus marinus*. Lengths were measured from the anterior end of the head to distal end of the telson (white dashed line).

2.2.2 Parasite prevalence and screening

A parasite prevalence study occurred over an 18 month period between the months of January 2010 to June 2011. To assess parasite prevalence and seasonality, 20 adults from each sex were selected from each monthly sample and stored in 70% ethanol at -80^{o}C. When samples were processed, the gonads and all muscle tissue was dissected and removed from the animal and washed with distilled water. The tissue was then placed in a 200µl of 5% Chelex solution (Sigma-Aldrich, UK) and homogenised using a disposable pestle (Fischer, UK). The solution had 3.75µl of proteinase K added, subsequently vortexed and incubated at 52^{o}C for a minimum of 4 hrs. The samples were then centrifuged at high speed (14000 g) and all supernatant was aspirated and the tissue homogenate discarded. The supernatant was incubated at 99^{o}C for 15 minutes to heat deactivate the proteinase K. A phenol/chloroform clean up step was then performed. Each sample was made up to 200 µl with dH_2O and 200 µl of a phenol solution consisting of 1 ml of phenol and 45 µl of buffer (Sigma-Aldrich, UK) was added and vortexed. Samples were then centrifuged at high speed (14000 g) for 2 minutes and the top layer (roughly 200 µl) was kept 200 µl of chloroform (sigma) was added, vortexed and centrifuged at 14000 g for 2 minutes and again the top layer was retrieved. To precipitate the DNA 500 µl of 100% ethanol and 20 µl of 3M sodium acetate at pH 7.2 were added to the sample, vortexed and frozen at -80 ^{o}C for 20 minutes. Samples were the centrifuged at 14,000 g for 15 minutes. All liquid was then removed and the pellet was air dried for 30 minutes to remove remaining ethanol and resuspended in 50 µl of distilled water. The DNA quantity was then measure using a spectrophotometer (Nano-drop ND1000) and sub-samples were diluted to 10 ng/µl for all future polymerase chain reaction (PCR) experiments. All DNA samples were stored at -80^{o}C.

Samples were then pooled using 250 males and 250 females (20ng from each individual) from sampling months July 2010 to July 2011. These DNA pools were then used to verify presence of different parasite groups. The parasite groups chosen for study were known to infect *E. marinus* or other amphipods and prioritised by parasites known to be highly influential in the population dynamics of other invertebrate species. Parasite groups screened via PCR were microsporidia (VIF and 1342), acanthocephalan (537F and 1133R), trematode (18SF and Trem18SR1),

Wolbachia (Wol16SF and Wol16SR), and Paramyxea (Par18sf and Par18sr) (see Table 1).

All PCR reactions were performed in 25 µl reactions containing 2.5 mM $MgCl^2$, 0.25 mM each deoxynucleotide, 0.5 mM each primer, 1 unit Taq DNA polymerase, 1 x buffer and 1µl (10ng) of template DNA. To check the quality of all DNA samples, amplification of the GAPDH gene was used as a control. PCR product size was visualised under a UV transilluminator using 1.2% agrose gel electrophoresis at 150 V for 20 mins with ethidium bromide using DNA size standards. Thermal cycling conditions for all PCR reactions detailed in Table 1. All PCR reactions were control verified using a no template control (NTC) and a positive control for all parasite groups. From this initial general screen we could isolate parasites infecting the Portsmouth population. The amplified rDNA regions were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the Sanger method (Souce Bioscience) before a BLAST analysis was performed against sequences stored in GenBank (NCBI, www.ncbi.nlm.nlh.gov). Subsequently, individual infection of up to 20 males and 20 females was then considered over the months to measure prevalence and seasonality of the parasites present over an 18 month period (Jan 10 to Jun 11).

Gene	Primer	Primer Source	Sequence 5' end to 3'	Thermal cycle
E. marinus Gapdh	GapdhF	Yang et al (2010)	ATAGTGTCCAACGCCTCCTG	94°C (4 min), 35 cycles of 94°C (30s), 60°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
	GapdhR		CCAGTGGAGGATGGAATGAT	
Trematode 18S	18SF	Near et al (1998)	AGATTAAGCCATGCATGCGTAAG	94°C (4 min), 35 cycles of 94°C (30s), 61°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
	Trem18SR1	Guler	GCCAACGGATGAACCATCGGCA	
Acanthocephala 18S	537F	Near et al (1998)	GCCGCGGTAATTCCAGCTC	94°C (4 min), 35 cycles of 94°C (30s), 59°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
	1133R		CTGGTGTGCCCCTCCGTC	
Wolbachia 16S	Wol16SF	Pourali et al (2009)	CATACCTATTCGAAGGGATAG	94°C (5 min), 38 cycles of 94°C (45s), 55°C (45s), and 72°C (45s), a final incubation of 5 min (72°C).
	Wol16SR		AGCTTCGAGTGAAACCAATTC	
Microsporidia 16S	VIF	Weis et al (1994)	CACCAGGTTGATTCTGCCTGAC	94°C (5 min), 42 cycles of 94°C (45s), 62°C (45s), and 72°C (1.45min), a final incubation of 5 min (72°C).
	1342	Adapted from McClymont et al. (2005)	ACGGGCGGTGTGTACAAGGTACAG	
D. duebenum 16S	DMR	Yang et al (2010)	GATTTCTCTTCCGCAATACCAAT	94°C (5 min), 42 cycles of 94°C (45s), 60°C (45s), and 72°C (45s), a final incubation of 5 min (72°C).
D. berrilonum 16S	BMR		GATTTCTCTTCCGCAATACAGA	
Paramartelia 18S	Par18SF	Short et al (2012)	CCAAACCAAACGATCGAAGT	94°C (5 min), 42 cycles of 94°C (45s), 63°C (45s), and 72°C (45s), a final incubation of 5 min (72°C).
	Par18SR		GGGCGGTGTGTACAAAG	

Table 1: Primer name, the target gene, source of primer, the primer sequence and PCR thermal cycle conditions for the parasite identification screening for the Langstone Harbour, *E. marinus* population.

2.2.3 Statistical analysis

Relationships between biological parameters (*E. marinus* abundance, reproductive output etc) and environmental data (temperature and salinity) were investigated using multiple regression analysis. Relationships between two biological parameters such as host abundance and parasite prevalence were statistically analysed using Pearson's correlation coefficients. Proportional analysis of parasite prevalence was conducted using a chi-square test. All analyses were conducted using the statistical software package (SPSS17).

2.3 Results

2.3.1 Population study

Monthly average densities of *E. marinus* ranged from 5.8 to 97.0 individuals per m^2 with a mean annual density $\pm$ standard error of 38.6 $\pm$ 5.6 individuals per m^2 (see Figure 10). The highest densities were observed during the summer months with peaks in the first and second year of study in June 2010 (74.6 individuals m^2) and in July 2011 (97.0 individuals m^2), respectively. Lowest densities were observed in the winter months, in particular January 2010 (5.8 individuals m^2) and February 2011 (10.2 individuals m^2). Regression analysis indicated over the two year period there was a significant relationship between the monthly densities of *E. marinus* and the mean monthly sea temperature ($P = 0.008$; $R = 0.591$; df = 1; F = 10.278; Figure 11). Multicollinearity analysis was conducted and indicated a low association in temporal monthly samples (Tolerance = 0.867; VIF = 1.157) and a slight serial correlation was observed (Durbin-Watson = 1.341) indicating the presence of temporal autocorrelation and that samples may not be fully independent. Seawater temperature slightly varied seasonally between the two years, however, both years presented a similar pattern. No significant relationship was observed between monthly densities of *E. marinus* and mean monthly salinity ($P = 0.212$; $R = 0.300$; df = 1; F = 1.685).

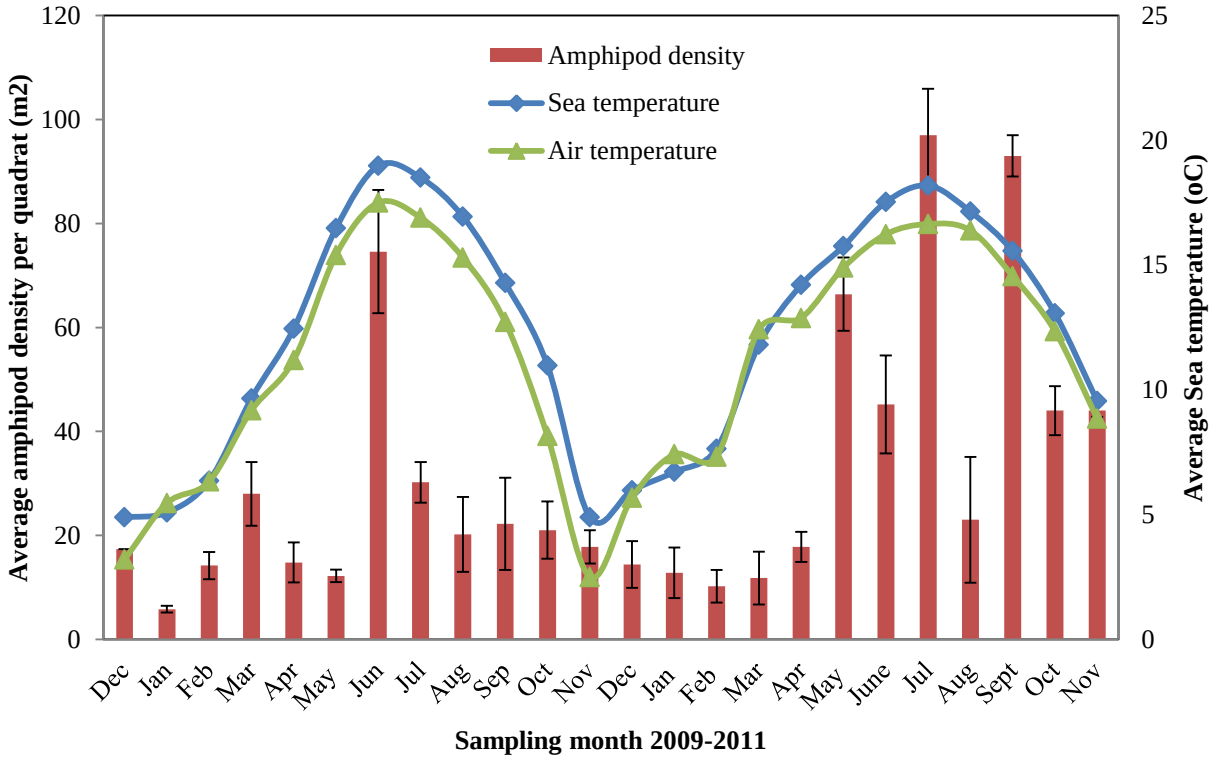

Figure 10: Seasonal variation in the density of *Echinogammarus marinus* (bar) and average monthly sea temperature (line) provided by CHIMET weather station. Error bars to one standard error.

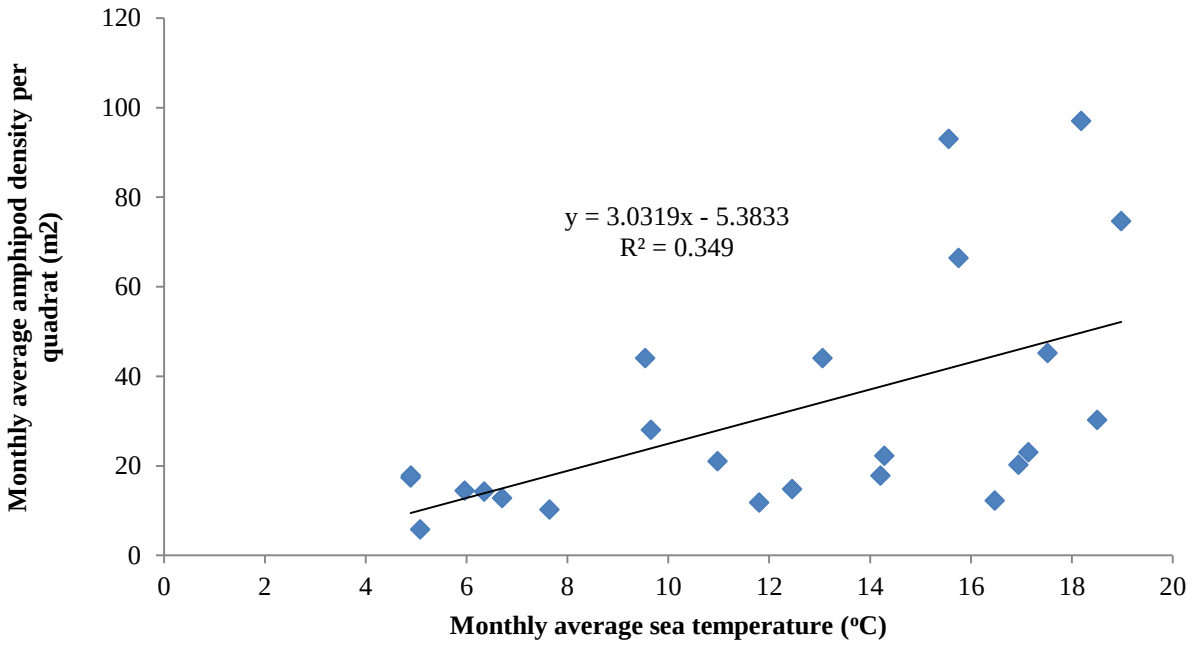

Figure 11: Linear relationship between monthly mean temperature and *Echinogammarus marinus* density from Langstone Harbour, Portsmouth (UK). Amphipod density from field data 2009-2011 and temperature data (CHIMET).

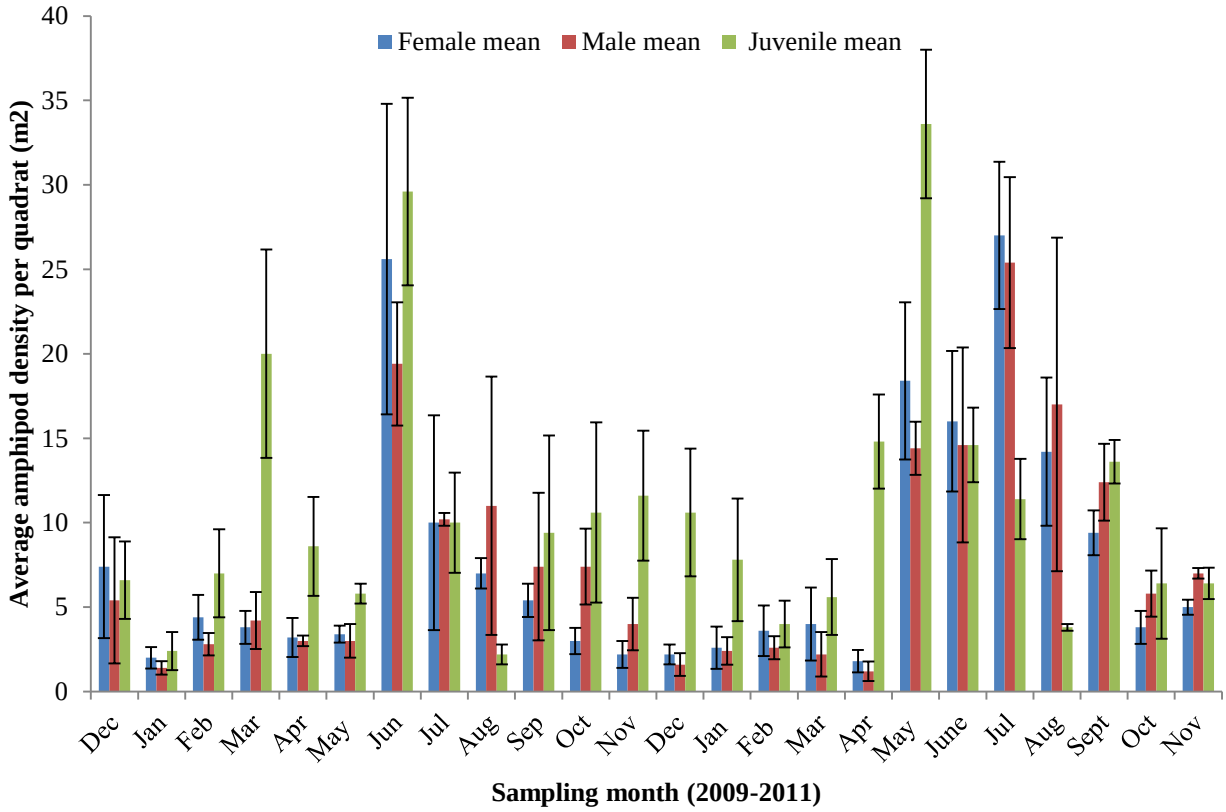

Figure 12: Population structure of juvenile, female and male *Echinogammarus marinus* in the Langstone Harbour, Portsmouth population during Dec 09 to Nov 11.

During the two year field study 3111 *E. marinus* were collected, measured and if the individual's gender could be morphologically distinguished, sexed. The total number of juveniles collected was 1301 and total adults collected were 1810, of which 910 were males and 900 were female. The population exhibited a 1:1 overall sex ratio, however, seasonal sex bias was present (see chapter three for further details). The juvenile population peaked in the early summer months (June 2010 and May 2011) (see Figure 12). Size-frequency distributions were examined to interpret the population structure at Langstone Harbour (see Figure 13). Amphipods taken from the population showed a mean length of 1.17 $\pm$ 0.01 cm ranging from 0.24 to 2.88 cm. Juveniles (small individuals) constituted 42% of the total population. Females could be morphologically identified at smaller lengths than males, and males grew larger than females. Males constituted higher size classes, whereas females were identified in the lower size classes. Female and male mean lengths were 1.53 $\pm$ 0.01 cm and 1.74 $\pm$ 0.01 cm, and the size range was 0.72 to 2.74 cm and 0.91 to 2.88 cm, respectively. Juvenile length ranged from 0.24 to 1.14 cm with a mean length of 0.52 cm (see Figure 13). Individuals showed sexual dimorphism over 1 cm (see Figure 8) and sexual maturity was reached by 0.92 $\pm$ 0.03 cm. External intersexuality was relatively low within the population the overall mean percentage of intersex in the sample population was 2.8 $\pm$ 0.6 %, over the two year sampling period this ranged from 0 to 9.7%. Although, intersex levels fluctuated, no obvious trends were observed.

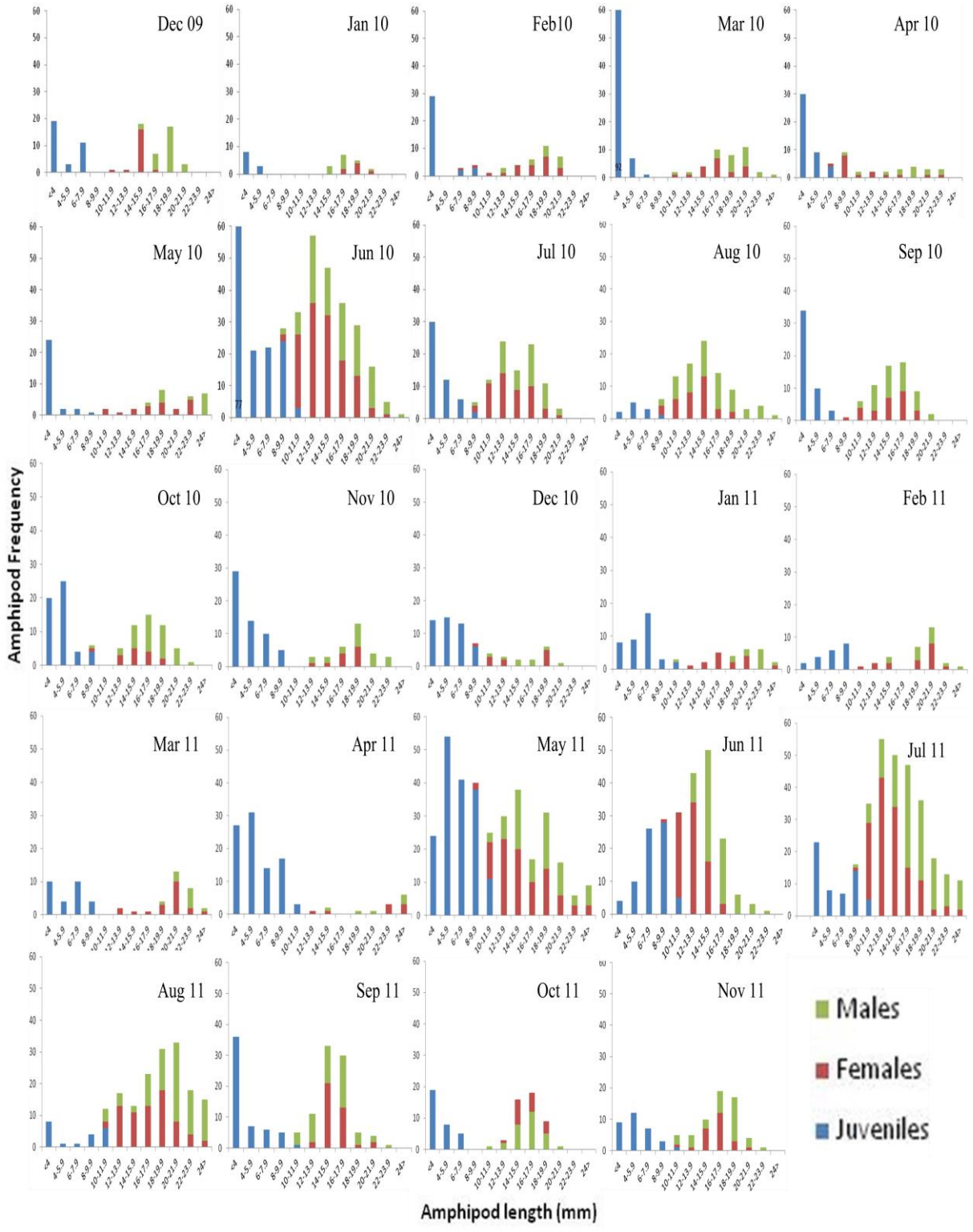

Figure 13: Length/frequency data of juvenile, female and male *Echinogammarus marinus* in the Langstone Harbour, Portsmouth population during December 2009 to November 2011.

E. marinus females were found to be ovigerous throughout the year, however, reproductive output fluctuated quite considerably (see Figure 14). For example, the mean percentage of ovigerous females was 40.0 $\pm$ 5.0 % and ranged from 5.7% (August 2010) to 88% (May 2010). Seasonal patterns were not mirrored over the two year period with peaks in the percentage of ovigerous females being observed throughout the year, in particular May 2010, January to March 2011 and October 2011 which correlated with peaks in the number of juveniles observed a couple of months later (see Figure 12). The data also reveals a reduction in reproductive output in August 2010 and July 2011. Regression analysis revealed a relationship between percentage of ovigerous females in the population and temperature, although this was not significant (P = 0.060 R = 0.440, df = 1, F = 4.073). No relationship was observed with salinity (P = 0.839 R = 0.050, df = 1, F = 0.043).

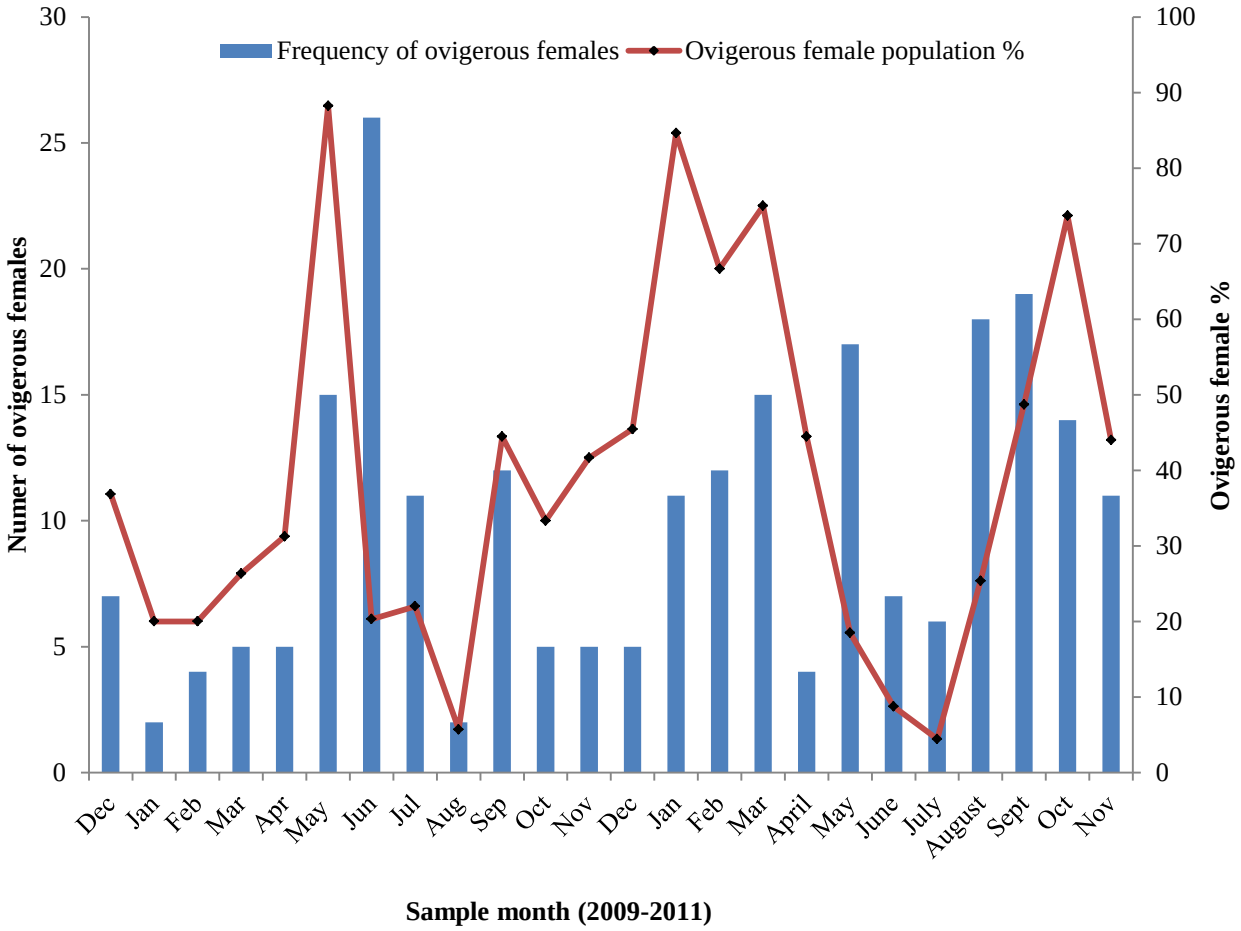

Figure 14: Frequency of ovigerous females and percentage of ovigerous female within the female population (number of ovigerous females/the total number of females in the monthly sample)*100, categorised by either egg bearing or juvenile bearing, from Langstone Harbour (Portsmouth, UK) between December 2009 and November 2011.

2.3.2 Parasite prevalence and screening

From the initial general parasite screen from the pool of 500 *E. marinus* individuals, microsporidian and trematode parasite species were identified from the Langstone Harbour, Portsmouth population. No infection from Paramyxea, *Wolbachia* or Acanthocephala species was observed. From past work, two microsporidia species have already been connected with *E. marinus* (Yang et al., 2011, Short et al. 2012b). Therefore, the Langstone Harbour was subsequently screened for *Dictyocoela berillonum* and *Dictyocoela duebenum* to establish if these were the species infecting the population. The microsporidia species was identified as, *D. berillonum* and no infection of *D. duebenum* was observed. BLAST search analysis (NCBI) of trematode sequences indicated the trematode belonged to the Microphallidae family, although the exact species could not be identified (see chapter three for further phylogentic analysis). From the sequencing reads generated, there was no indication of mixed signal, suggesting an isolated trematode dominates the Langstone Harbour population.

Further to establishing which parasites infected Langstone Harbour *E. marinus* Population, individual infection rates of *D. berillonum* and the trematode were then recorded over an 18 month period during January 2010 to June 2011. The overall mean infection prevalence of *D. berillonum* over the study period was 14.43$\pm$1.49 % and ranged from 5.13 to 29.41 %. There was no significant difference in infection prevalence between males and females. Infection of the population peaked in both February (2010) and May (2011) with a crash in infection rates in March 2010 and 2011 and in January 2011 (see Figure 15). There was no correlation (Pearson's correlation coefficient) seen between the microsporidia prevalence and abundance of host (R = -0.258, P = 0.301), host intersexuality (R = 0.057, P = 0.821), or percentage of ovigerous females (R = 0.267, P = 0.271). Regression analysis failed to detect a significant relationship between microsporidia prevalence and sea temperature (P = 0.510 R = 0.166, df = 1, F = 0.454) or salinity (P = 0.174 R = 0.335, df = 1, F = 2.023).

The overall mean infection prevalence of the trematode over the study period was 40.2 $\pm$ 3.6 % and ranged from 17.5 to 70.0 %. There was no difference between infection prevalence in males and females. Infection within the population peaks in February and July for both 2010 and 2011, with a crash in infection rates in October 2010 (Figure 16). There was no correlation observed between the trematode prevalence and abundance of host when directly comparing the months (Pearson's correlation coefficient; R= -0.330, P = 0.168). Trematode prevalence can have a delayed effect on host abundance, trematode prevalence was aligned with host abundance +1 month under these conditions a significant relationship was observed (Pearson's correlation coefficient; R= -.461, P = 0.047) (see Figure 17). Regression analysis failed to detect a relationship between trematode prevalence and sea temperature (P = 0.135 R = 0.582, df = 1, F = 0.315) or salinity (P = 0.282, R = 0.260, df = 1, F = 1.236).

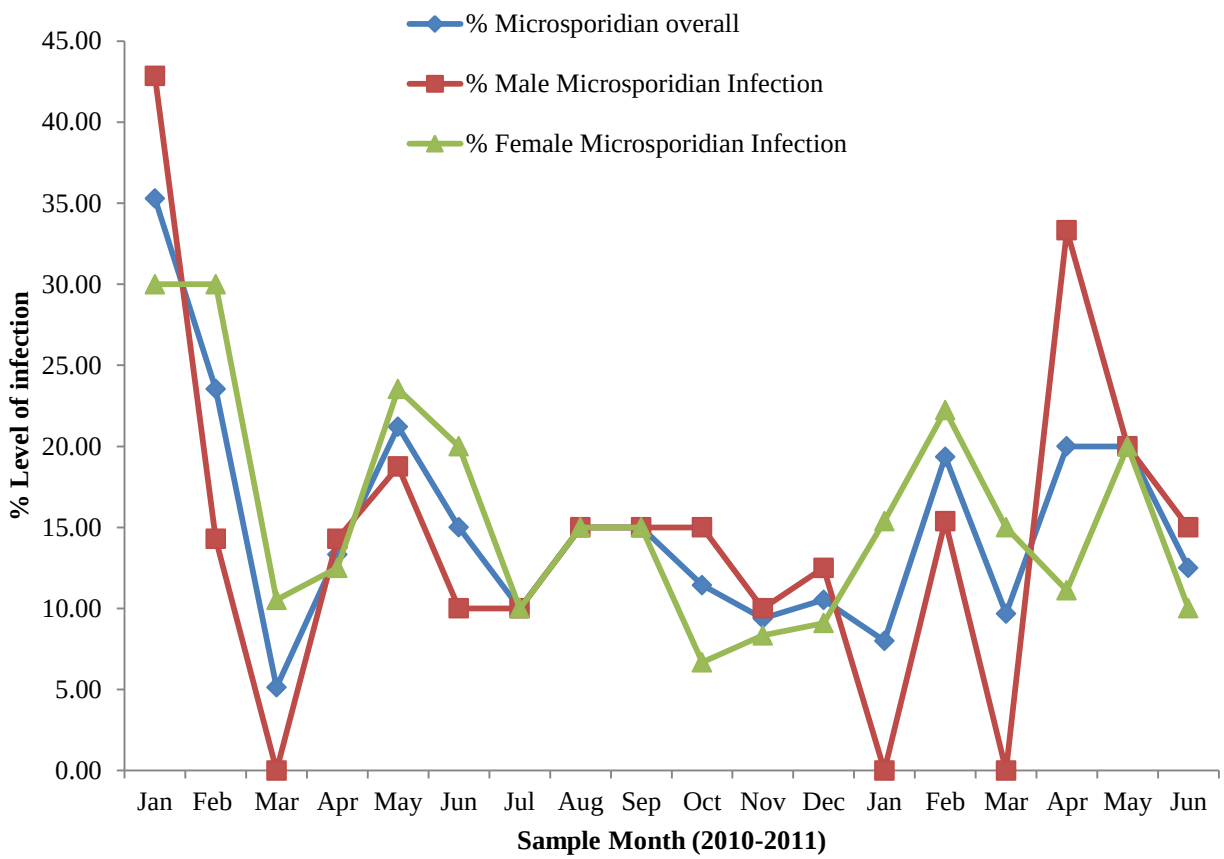

Figure 15: Linear relationship between host (Echinogammarus marinus) and parasite, *Dictyocoela berillonum* (Microsporidia) from Langstone Harbour, Portsmouth (UK) from January 2010 to July 2011.

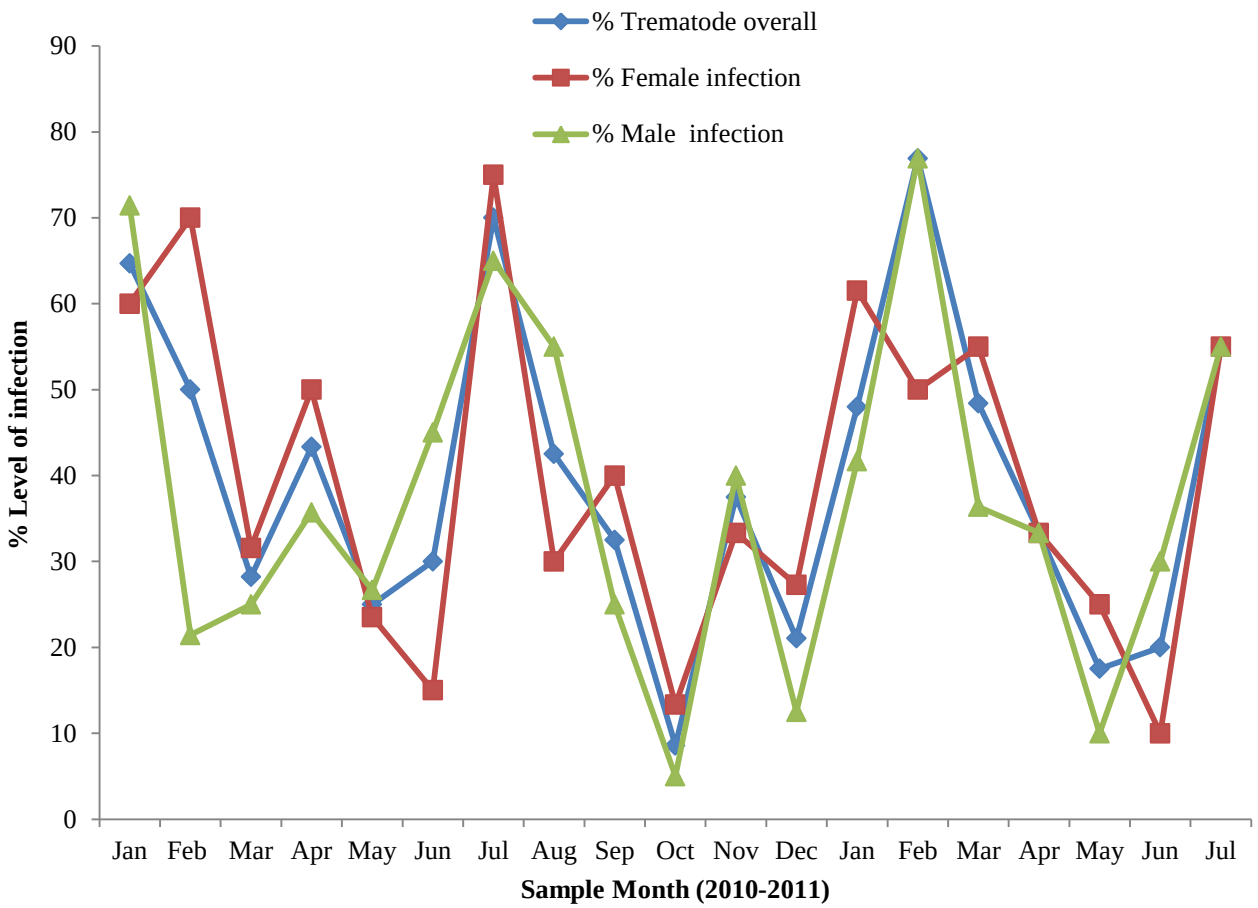

Figure 16: Seasonal prevalence of trematode parasites within *Echinogammarus marinus* population from Langstone Harbour, Portsmouth (UK) from January 2010 to June 2011.

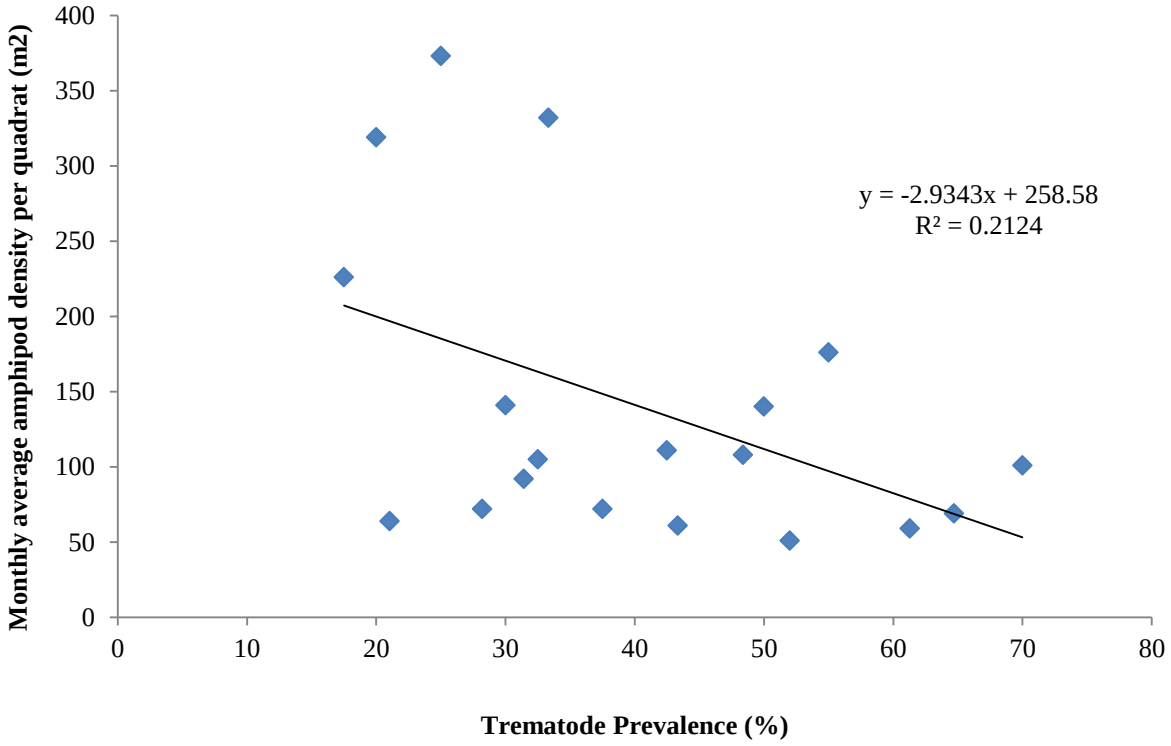

Figure 17: Linear relationship between *Echinogammarus marinus* density from Langstone Harbour, Portsmouth (UK) and prevalence of a trematode parasite. Data obtained from field study during 2009-2011.

Trematode metacercariae could be clearly seen within *E. marinus*, this is due to the immune response (melanisation) that encapsulates the metacercariae, making the cyst appears dark brown (See Figure 18A). While amphipod individuals were being dissected for DNA extraction, it was observed that some metacercariae do not develop this immune capsule (See Figure 18B). Records of individuals with visual cysts were recorded in this study. The animals that were visually infected matched up with a positive PCR result for the presence of trematode. However, the molecular screening also gave positive results for individuals that had no visual metacercariae. The PCR method suggested an infection prevalence of 40.2 $\pm$ 3.6 %, whereas visual identification infection through cyst prevalence was 28.63 $\pm$ 3.8%.

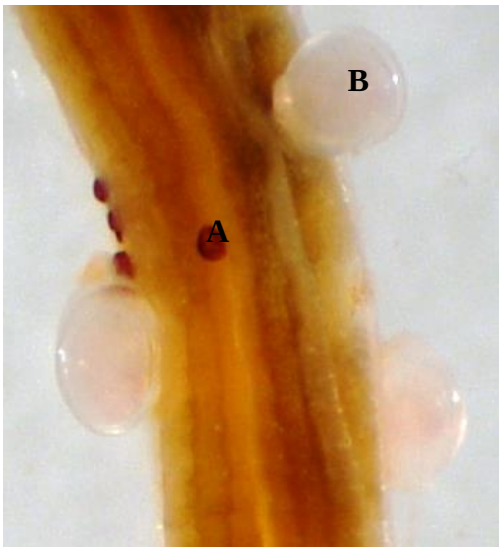

Figure 18: *Echinogammarus marinus* hepatopancreas with trematode metacarceriae cysts attached with an immune response that visually appear brown (A) and cysts that have not developed an immune response that have a white, opaque appearance (B).

There was no observed interaction between the infection ratios of the microsporidian and trematode parasites. Peaks and crashes in the infection prevalence of the two parasites did not correlate with each other (R = -0.181, P = 0.564) (see Figure 19). The screen for microsporidia and trematode within the population recorded an overall 10.5% and 28% infection prevalence, respectively. Statistical analysis indicated a significant difference between the two parasite species infection prevalence in *E. marinus* (X^2 = 76.0494 ; df = 1 ; P = < 0.0001). There was no statistical significance (X^2 = 0.7802 ; df = 1 ; P = 0.3771) between the prevalence of microsporidian in the total population (10.5%) than those also infected with the trematode (12.9%). Equally there was no statistical significance (X^2 = 0.7802 ; df = 1 ; P = 0.3771) between the prevalence of trematode in the total population (28.7%) than those also infected with the microsporidia (34%) suggesting that neither parasite influence the prevalence of the other.

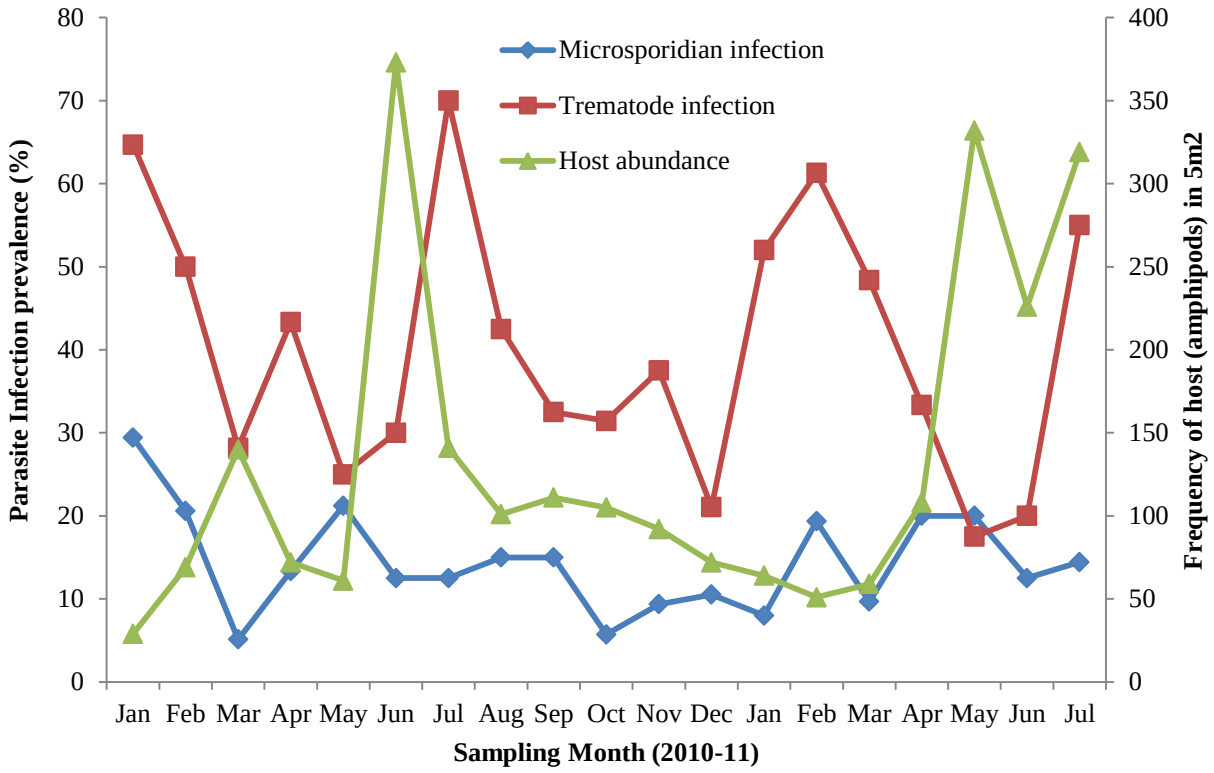

Figure 19: Overall infections rates of trematode and microsporidia parasites in *Echinogammarus marinus*.

Microsporidia only infected	Trematode only infected	Co-infection	Total infected	Uninfected
3	9	2	14	12
4	14	3	21	27
1	10	1	12	37
3	12	1	16	26
5	6	2	13	26
4	11	1	16	35
1	24	4	28	35
2	13	4	19	34
5	12	1	18	34
1	2	1	4	33
2	11	1	14	29
2	4	0	6	17
1	12	1	14	23
3	16	3	21	25
1	13	2	16	28
3	5	0	8	12
8	7	0	15	32
4	7	1	12	35
53	**188**	**28**	**267**	**500**

Table 2: Infection rates of trematode and microsporidian parasites in *Echinogammarus marinus* Langstone Harbour population with single and co- infection rates revealing low association between the two parasite groups.

2.4 Discussion

The *E. marinus* population density at Langstone Harbour showed clear seasonal variation, a result consistent with other *E. marinus* populations (Maranhao et al., 2001, Vlasblom 1969). The highest densities were observed during the summer months with the lowest densities being observed in the winter months. There was a significant correlation between the monthly densities of *E. marinus* and the mean monthly sea temperature. In addition, despite not being significant, a relationship between the percentage of ovigerous females and temperature was also observed. Environmental parameters, such as temperature, are highly influential factors in the reproductive processes of amphipods (Bettison and Davenport, 1976, Maranhao et al., 2001, Maranhao and Marques, 2003). *E. marinus* field studies have also demonstrated that the percentage of egg bearing females, population density, sex ratio, egg volume and fecundity are all influenced by salinity and temperature (Maranhão et al., 2001). The findings in this study confirm that *E. marinus*, like many amphipod species, are sensitive to environmental parameters.

Sexual activity and recruitment occurs throughout the year at the Langstone Harbour population which supports previous studies of *E. marinus* (Maranhao et al., 2001) as well as, other amphipod populations (Costa and Costa, 1999, Covi and Kneib 1995, Drave and Arias, 1995, Moore and Wong, 1996). It was therefore consistent to find no obvious cohorts in the Langstone Harbour population. Peaks in the juvenile population were observed in the early summer months, but decreased in the winter months. This appears to correspond with other *E. marinus* population studies in different regions (Maranhao et al., 2001, Vlasblom, 1969). The seasonal patterns of ovigerous females fluctuated over the two year period with peaks that were not seasonally distributed, with the exception of the late summer months in which a reduction in reproductive output was observed in both years. The population exhibited a 1:1 overall sex ratio; however, a seasonal sex bias was present, which will be discussed in detail in chapter four.

Generally in amphipod species, males have larger size ranges because they reach a larger maximal size (Borowsky, 1984). Sexual size dimorphism is ubiquitous among gammarids and is linked with fitness and fecundity (McCabe and Dunn, 1997). In this study, the female and male size range was 0.72 to 2.74 cm and 0.91 to 2.88 cm respectively. Females could be morphologically recognised at a smaller size than the males and the maximum length of the amphipods did not differ greatly between genders. Therefore, the larger size range observed in females was probably due to early stage brood plates being more noticeable than early stage genital papillae. Another sampling bias that may have occurred relates to the collection of juveniles in mud and seaweed, which can often be difficult due to their small size. Juvenile numbers could have been underestimated, despite that samples were sieved and collection was thorough.

Little interaction between environmental parameters and *E. marinus* data was observed, with the exception of some interaction between sea temperature and the percentage of ovigerous females in the population, as well as temperature and *E. marinus* abundance. Intertidal animals are well adapted to swings in environmental conditions due to the nature of their habitat. Environmental conditions are not as varied in the south of England compared with locations hosting other *E. marinus* populations (Maranhao et. al., 2001). Further investigation into other parameters that are known to affect *E. marinus* or amphipod species such as food availability (green macro algae biomass) (Drake and Arias, 1995, Maranhao et al., 2001, Pardal et al., 2000), and predator abundance (seasonal bird abundance) would possibly elucidate further population effects on the Langstone Harbour population.

The study of the two parasites identified to infect the Langstone Harbour population revealed that while the trematode fluctuates greatly throughout the study period, the microsporidia presented a more consistent infection prevalence. These parasite groups have very different transmission strategies and life cycles which could account for the differences in the temporal infection prevalence. Trematodes are horizontally transmitting parasites that have multi host life cycles and are known to

alter their amphipod host's behaviour to increase their likelihood of being predated on by birds or fish, thereby reaching their definitive host (Poulin and Cribb, 2002). Parasite and host fitness are conflicting and often the parasite has pathogenic or detrimental effects upon the host. Microsporidia are obligate intracellular parasites that use vertical and horizontal transmission (Dunn and Smith, 2001, Terry et al., 2004). *D. berillonum* is a vertically transmitting parasite (Yang et al., 2011), although, as there is no extreme female bias or female biased infection, data from this study suggests that this is not a feminiser. This supports previous studies that examined *D. berillonum* infection in amphipods (Terry et al., 2004, Yang et al., 2011).

Since vertically transmitted parasites are highly invested in host health because of their requirement for successful host reproduction, they can be in direct conflict with horizontally transmitted parasites. This study revealed no obvious interactions between the trematode and microsporidian species infecting the Langstone harbour population. Haine et al. (2005) investigated how horizontal parasites, such as acanthocephalans, can affect vertically transmitting microsporidian parasites and how or if, coexistence can occur despite their different modes of transmission. It was suggested that two conflicting parasites competing for the same host might adopt either a strategy of avoidance or sabotage. However, co-infection levels in this study were as expected given the individual infection prevalence of the two parasite species. High co-infection prevalence of the two parasites infecting the *E. marinus* population at Langstone Harbour would be detrimental for one or possibly both parasite species as they would be directly competing for opposite strategies. Therefore, further investigation into whether the co-prevalence of the two species in a single individual reduces parasite fitness or lessens their effects could reveal possible parasite competition interactions

Seasonal variation of the trematode parasite was more dramatic compared with the relatively consistent infection rates of the microsporidian. Meissner (2001) recorded seasonal patterns of trematode prevalence in the amphipod *Corophium volutator* that showed relatively low variation in seasonal prevalence, with lowest prevalence being observed in spring and early summer, when juvenile numbers are at their highest.

Infection prevalence was observed to steadily increase during the summer and peaked in the late summer and autumn months (Meissner, 2001). This study revealed trematode fluctuations correlated with host abundance, rather than temperature. No relationships were observed between trematode abundance and any environmental parameters studied, which is consistent with other trematode studies that also have shown no environmental influences (Lagrue and Poulin, 2008). Understanding parasite influences upon their hosts and the effects on population dynamics can be problematic due to the numerous influencing factors at play. This complexity can increase if the parasite has multiple hosts. Mud snails (Hydrobia) were abundant at the sample site and it is extremely likely to be the first intermediate host within the trematode infecting *E. marinus*, as they represent frequent intermediate hosts (Bordalo et al., 2011, Zander et al., 2000). In addition, multiple bird species inhabit the area and are a good candidate for the definitive host of the trematode found in *E. marinus*. It is probable that the trematode prevalence within *E. marinus* is strongly dependent upon the other hosts necessary for its lifecycle as well.

Trematode parasites are well documented to impact the population dynamics of various hosts due to their behaviour altering capacity (Damsgaard et al., 2005). Increased surface activity has been observed in a population of *C. volutator* that was infected with several microphallid trematode species, an activity linked with the local extinction of the population (Damsgaard et al., 2005). In this study, a significant correlation between host abundance and trematode prevalence was observed. Great fluctuations in the trematode infection prevalence could be evidence of a build up of infected individuals that then go through mass mortality over time, resulting in the appearance of reduction in infection prevalence within the population. Further investigation is needed to determine whether the mass mortality is through behavioural manipulation causing predation or pathogenic induced mortality. However, data suggests this microphallid parasite has the capacity of having great influence on this *E. marinus* population. This also emphasises how important the awareness of parasite prevalence and abundance is to understanding population dynamics of any species. (Poulin and Mouritsen, 2006).

The prevalence of microsporidia fluctuated far less than the trematode; however the microsporidia prevalence does dip during certain months. The level of infection within *G. duebeni* is closely associated with parasite burden and efficiency of transmission (Dunn and Hatcher, 1997b). In amphipod species abiotic factors such as temperature and salinity can highly influence the parasite transmission (Dunn and Hatcher, 1997a, Dunn et al., 2006b). Although, this study observed no relationship in the microsporidia prevalence with mean monthly sea temperature or salinity. The fluctuations observed could be particular temperature ranges and salinities restricting transmission rates at certain points of the year. This is probably due to temperature and salinity ranging between levels within the parasite can replicate and transmit which reflects the some what consistant prevalence. Microsporidia transmission is passed on to the next generation constantly as the *E. marinus* population reproduces throughout the year.

Trematode metacercariae could be visually seen within *E. marinus*, due to the immune response that encapsulates the metacercariae. It was observed that some metacercariae do not develop this immune capsule. This could either be due to the metacercariae recently entering the amphipod host, therefore, not giving a sufficient amount of time for the development of a response, or that more substantial well developed metacercariae have a greater tolerance to the host immune system than the smaller sized cysts that have larger surface to volume ratio, perhaps making a host immune system response to be successful. Consistent with this was the observation that, generally, the metacaceriae cysts that had not developed an immune response were larger. Visual identification of infection is how many trematode studies measure prevalence in the field (Lagrue and Poulin, 2008). The animals that were visually infected matched up with a positive PCR result for the presence of trematode. However, the molecular screening also gave positive results for 11% more individuals that had no visual metacercariae. The most probable explanation is that these individuals possess metacercariae that have recently entered the host and have not yet induced a sufficient immune response making them less visually

obvious. PCR methods are a more accurate way of establishing prevalence within this parasite for future work as a result of its increased sensitivity.

This study has given insight into the Langstone Harbour population highlighting multivoltine life cycle, in which breeding is not seasonally restricted and recruitment occurs throughout the year. *E. marinus* abundance and reproductive output was influenced by temperature, although no other correlations with the environmental parameters investigated were observed. The population was found to be infected by the microsporidian, *D. berillonum* and an undescribed trematode belonging to the Microphallidae family out of the parasite groups that were investigated. This study did observe large seasonal variability in trematode abundance, whereas *D. berillonum* showed a more consistent infection rate. The trematode prevalence showed a significant correlation with host abundance. Although neither parasite species showed any effects on infection prevalence of the other parasite, or showed any sex bias in their infection rates. This study has highlighted several new paths of research within this widespread amphipod species, as well as providing a basic understanding of the population dynamics at Langstone Harbour, Portsmouth. In particular the work has shown the importance of parasitology work in population dynamics research.

3. The identification and effects of digenean trematode parasites that infect *Echinogammarus marinus*

3.1 Introduction

The first known example of a trematode parasite infecting *Echinogammarus marinus* was revealed during a screen to identifying parasite groups thought capable of greatly influencing host population dynamics (Chapter 2). The screen revealed a high prevalence of metacercariae in the body cavity and brain. The main focus of this chapter is to determine the trematode species that infects *E. marinus* populations and the possible effects that they may induce.

Among intertidal animals, trematodes are the most common parasite and consist of an estimated 25000 species, many of which have yet be described (Mouritsen and Poulin, 2002). The class Trematoda comprises of two subclasses, Digenea and Aspidogastrea, that forms part of the Neodermata clade (platyhelminthes) (Littlewood et al., 1999). Trematodes are obligatorily parasites that have complex life cycles, infecting up to four hosts, taking many distinct forms and can infect their hosts in a variety of ways (Esch et al., 2002). Their life cycle comprises both free living and parasitic stages and can incorporate asexual and sexual reproduction (see Figure 20), with gammarids generally acting as intermediate hosts within the trematode life cycles. The complexity of a multi host life is a risky strategy as any interruption in life cycle steps would result in incompletion to the final goal, sexual reproduction within the final host. This has resulted in various adaptations to maximise success and to counteract the risks (Poulin and Cribb, 2002). For example, some species have truncated their life cycle by removing hosts thus reducing the number of transmitting events between hosts (Poulin and Cribb, 2002, Rauch et al., 2005). Reducing the number of hosts seems like the obvious answer to decreasing the risk of life cycle incompletion, many species still possess three hosts in their life cycle.

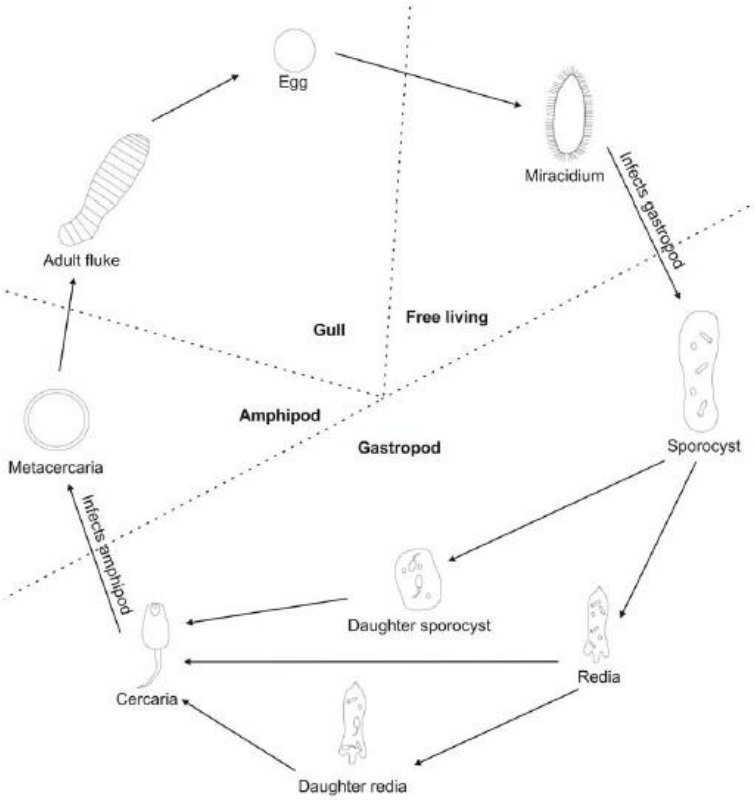

Figure 20: The complex life cycle of microphallid trematodes infecting amphipods (from Gates 2006).

Utilising multiple hosts facilitates an increase in life stages, this can increase the potential for higher growth and fecundity (Parker et al., 2003), increase probability of locating a mate (Brown et al., 2001), higher transmission rates, longer life span (Choisy et al., 2003, Parker et al., 2003), as well as increasing clone intermixture, thus decreasing the risk of mating between genetically identical individuals (Keeney et al., 2007). Another strategy adopted is to produce high numbers through life stages via asexual production and high adult fecundity. For example, sporocysts can produce 500000 cercariae within a host per day (Poulin and Cribb, 2002; Haas, 2003). Within the micracidium stage the larvae have evolved to utilise chemotaxis and chemokinesis to detect the intermediate host (Haas, 2003). Lastly, the presence of metacercariae can manipulate the intermediate host activity (behaviour) such that it increases the chance of trophic transmission to the definitive host (Bethel and Holmes, 1973, Camp and Huizinga, 1979, Combes, 1991).

Behaviour alterations observed in parasite infected animals can be through a side effect of infection or pathologically induced behaviour modification due to the presence of the parasite (Poulin, 1995). Debilitating parasites can induce multiple alterations that can substantially modify the biology of the host leading to population level effects (Thomas et al., 1995). The volume of metacercariae can increase 40-fold during microphallid development causing a massive shift in the allocation of host resources which, in turn, can cause malnutrition due to increased food requirements, increased oxygen demand and obstruction of locomotory apparatus (Benjamin and James, 1987, Galaktionov et al., 1996). Although not a direct manipulation in host behaviour an increased food demand could cause host relocation, thereby changing 'normal' behaviour (Damsgaard et al., 2005), which consequently could increase chances of predation or could have no adaptive strategy and simply be an arbitrary side effect.

Behavioural alterations induced by some trematode species do not always exhibit the same effects on different hosts. The trematode *Maritrema novaezealandensis* is known to cause reduced activity in the amphipod *Paracalliope novizealandiae*, which is thought to be induced by the pathology of the parasites presence (Leung and Poulin, 2006). However, the shore crab, *Macrophtalmus hirtipes* infected with the

same parasite showed no altered behaviour (Martorelli et al., 2004). This could be due to another parasite masking effects, or differences in the biology between amphipods and crabs, possibly amphipods are more susceptible to manipulation in their biology or behaviour manipulation is easier to observe in amphipods due to their greater mobility. Similarly, the trematode, *Microphallus papillorobustus*, infects two sympatric amphipod species, *Gammarus insensibilis* and *Gammarus aequicauda* (Helluy, 1983a). However, while infected *G. insensibilis* shows behaviour manipulation increasing the likelihood of avian predation, *G. aequicauda* only exhibits this behaviour when infected during juvenile stages and infection as an adult shows no behaviour modification (Helluy, 1983a, Helluy, 1984, Helluy and Thomas, 2003).

The trematode, *Maritrema subdolum* is known to have close association with gammarids that are also infected with *M. papillorobustus*, a microphallid that encysts within the head (Helluy, 1982). *M. subdolum* remain within the abdomen region, and are believed not to be involved in the behaviour manipulation associated with *M. pabillorobustus* (Kostadinova and Mavrodieva, 2005). These closely related species, infecting similar hosts, do not share the same manipulating effects, although their co-presence within gammarids does indicate a possible hitch-hiking strategy (Thomas et al., 1997). Cercariae of *M. subdolum* have greater mobility within the water column than those of *M. papillorobustus*, therefore can access gammarids already infected with a behaviour manipulating trematode and can increase their chance of transmission (Thomas *et al*., 1997; Thomas and Helluy, 2002). This indicates that even closely related species can differ in adaptive strategy.

Research into cerebral encysting trematodes that cause manipulation via neurological pathways has now been established (Helluy and Thomas, 2010, Shaw et al., 2009, Ponton et al., 2006). A well documented example are gammarids infected by *M. papillorobustus*, that cause a positive phototaxic and negative geotactic response, an abnormal behaviour pattern, considering the usual evasive manner observed in amphipods (Helluy, 1983a, Helluy, 1983b). Injection of serotonin in the hemocoel of gammarids also induces similar effects (Helluy and Holmes, 1989, Tain et al., 2006)., while other neurotransmitters investigated (GABA, noradrenaline, dopamine and

octamine) at similar concentrations, failed to induce an comparable response (Helluy and Holmes, 1989).

M. papillorobustus metacercariae migrate to their host's brain and encyst in the cerebral ganglia, specifically within the protocerebrum, the very front section of the brain responsible for all visual sensory input (Kostadinova and Mavrodieva, 2005; Thomas *et al*., 2000; Helluy and Thomas, 2003). Once inside the brain, serotonin levels become altered in specific regions, but most significantly a decrease of 62% was observed in the optic neuropils. The degeneration of discrete sets of serotonergic neurons in parasitised individuals was suggested to be the cause of the serotonin imbalance, which is thought to result in the aberrant photic behaviour (Helluy and Thomas, 2003). Mechanical displacement within the host brain can cause some alteration as well, with large cysts affecting important neuronal architecture in the small brain, particularly the adjacently located optic tracts (Helluy and Thomas, 2003). Recently, a study by Helluy and Thomas (2010) has shown that *M. papillorobustus* also induces a specific immune response, with neuro-inflammation being indicated by elevated levels of nitric oxide synthase and astrocyte-like glia being present at the host-parasite interface within the brain. The neuro-inflammation response in the host brain is suggested to play a role in the neuro-modulation and consequent behaviour alteration observed in previous studies.

The aims of this chapter are to identify the trematode species infecting *E. marinus* populations via molecular characterisation of the 18S, 28S and the ITS regions of the trematodes rDNA gene. Three *E. marinus* populations will be studied Langstone Harbour, Portsmouth; Inverkeithing, Scotland; and Loch fleet, Scotland, UK. The rDNA sequences of any trematode species infecting *E. marinus* can then be compared with published digenean parasite sequences and allow phylogentic analysis. The application of molecular tools to phylogenetically analyse trematodes has become widespread in recent years (Littlewood et al., 1999, Olson et al., 2003b, Tkach et al., 2003). However, relatively well studied trematode parasites still lack molecular data (e.g. *M. pabillorobustus*). *M. pabillorobustus* metacercariae rDNA will be sequenced due to its similarities with the trematode infecting the Langstone Harbour population. Both species have similar geographical range, utilise gammarids

as intermediate hosts, are morphologically alike, and encyst within the brain or attach to nerves in the thorax or abdomen. This study also aims to elucidate the adaptive strategy of the trematode infecting the Langstone Harbour population by conducting geo- and phototaxic behavioural assays to establish whether they induce behavioural alteration within *E. marinus*. On the basis of the findings of the behavioural assays, a molecular study will be performed to reveal whether there is any altered regulation of genes in infected animals to reveal possible mechanisms being affected in the hosts biology.

3.2 Methods

3.21 Trematode Identification

E. marinus were collected from beneath seaweed and rocks in the intertidal zone during low tide. Infected *E. marinus* individuals were taken from three different populations: Langstone harbour, Portsmouth (see Figure 7), Inverkeithing, Fife, Scotland (56°1’38”N 3°23’37”W) (see Figure 21) and Loch Fleet, Scotland (56° 05’ 35.7”N 3° 09’ 29.0”W) (see Figure 22). Samples from Loch Fleet were obtained from Dr Alex Ford (2006). *G. insensibilis* infected by *M. pabillorobustus* were collected in the south of France (Etang de Thau, 43°25'N, 3°35'E) and were kindly donated by Dr Frederic Thomas (University of Montreal). The parasite cysts from each population were dissected from their host. 20 individuals per host population were used to obtain enough parasite tissue and stored in 70% ethanol at -80°C. DNA extracted using the DNAeasy kit (Qiagen, UK) following the manufacturers protocol.

Figure 21: Illustration of the sample site for the collection of *E. marinus* at Inverkeithing, Fife, Scotland (56°1'38"N 3°23'37"W). Image was produced by Google Earth Software (2012).

Figure 22: Illustration of the sample site (red triangle) for the collection of *Echinogammarus marinus* at Loch Fleet, Scotland (56° 05' 35.7"N 3° 09' 29.0"W). Image was produced by Google Earth Software (2012).

The trematode DNA was amplified using general acanthocephalan primers of the 18s ribosomal gene (Near et al., 1998). (Initially the parasite cysts were morphologically identified as an acanthocephalan species.) Primers used to amplify the 18S gene were 537F and 1133R, 1073F and 18SR, 18SF and 549R to the conditions in accordance with Near et al. 1998 (see Table 3). To gain the complete 18S sequence primers were designed after initial sequencing (Trem18SR1 and 2). 18SF and Trem18SR1 were used in the amplification and the sequencing was conducting using the Trem18SR1 and 2 (see Table 3). Primers T28SF and T28SR were used to amplify the 28S region (Olson et al., 2003). To sequence the ITS region primers were designed specifically from the 3' end of the trematode 18S region (PITSF) and the 5' end of the 28S region (PITSR). Parasite and host sequences were aligned (ClustalW2) (www.ebi.ac.uk) and ITS primers were designed against parasite specific sequences to ensure no host template was amplified.

All primers were synthesised by Eurofins MWG Operon and all PCR product sizes were verified by electrophoresis on a 2% agarose gel using DNA size standards. DNA products was purified and eluted with the QIAquick Gel Extraction Kit (Qiagen, UK) gel purification kit following the manufacturer's protocol. The purified PCR products were subsequently sequenced using the Sanger method (Source Bioscience) and BLAST analysis on the newly generated sequences were performed against the NCBI (National Centre for Biotechnology Information, www.ncbi.nlm.nlh.gov) database to confirm the closest sequence annotation. Other trematode sequences were gathered from this database for phylogenetic comparison, which were then aligned (ClustalW2) and trimmed, a phylogenetic tree was constructed using the maximum likelihood method implemented by the PhyML (v3.0, www.phylogeny.fr) program, with the reliability of the branching being assessed using the bootstrap method (n = 100) (Dereeper et al., 2008).

Primer	Sequence 5' end to 3'	PCR Reactions- (25-µl) (Promega GoTaq®)	Thermal cycle
18SF	AGATTAAGCCATGCATGCGTAAG	2.5 mM $MgCl_2$, (x1) Buffer, 0.2 mM each dNTP's, 0.25 mM each primer, 1 units Taq DNA polymerase, 10ng gDNA	94°C (4 min), 35 cycles of 94°C (30s), 60°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
549R	GAATTACCGCGGCTGCTGG		
537F	GCCGCGGTAATTCCAGCTC	2.5 mM $MgCl_2$, (1x) Buffer, 0.2 mM each dNTP's, 0.25 mM each primer, 1U Taq DNA polymerase, 10ng gDNA	94°C (4 min), 35 cycles of 94°C (30s), 60°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
1133R	CTGGTGTGCCCCTCCGTC		
1073F	CGGGGGGAGTATGGTTGC	2.5 mM $MgCl_2$, (1x) Buffer, 0.2 mM each dNTP's, 0.25 mM each primer, 1U Taq DNA polymerase, 10ng gDNA	94°C (4 min), 35 cycles of 94°C (30s), 60°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
18SR	TGATCCTTCTGCAGGTTCACCTAC		
Trem18SR1	GCCAACGGATGAACCATCGGCA	2.5 mM $MgCl_2$, (1x) Buffer, 0.2 mM each dNTP's, 0.25 mM each primer, 1U Taq DNA polymerase, 10ng gDNA	94°C (4 min), 35 cycles of 94°C (30s), 60°C (30s), and 72°C (45s), a final incubation of 5 min (72°C).
Trem18SR2	TGGAGTTACCGCGGCTGCT		
T28SF	TAGGTCGACCCGCTGAAYTTAAGCA	2.5 mM $MgCl_2$, (1x) Buffer, 0.2 mM each dNTP's, 0.25 mM each primer, 1U Taq DNA polymerase, 10ng gDNA	94°C for 4 min, 40 cycles of 94°C (30s), 56°C (30s), and 72°C (45s),a final incubation of 5 min (72°C).
T28SR	GCTATCCTGAGGGAAACTTCG		
PITSF	GTTTCGACTGCTCGAGTGGTG	1.25 mM $MgCl_2$, (1x) Buffer, 0.2 mM each dNTP's, 0.25 mM each primer, 1U Taq DNA polymerase, 5ng gDNA	94°C for 4 min, 32 cycles of 94°C (45s), 59°C (45s), and 72°C (1.5 min), a final incubation of 5 min (72°C)
PITSR	AACAACCTGAACACCACATTG		

Table 3: Primer name, sequences and PCR conditions for trematode rDNA phylogeny analysis.

3.22 Behavioural effects

E. marinus were collected in Langstone Harbour, Portsmouth, UK at low tide from beneath seaweeds (mainly fucoids) and rocks. Individuals were then sexed and separated according to infection. Only male individuals were used, specimens were considered infected if they visually heavily infected with trematode cysts. All individuals were kept in a set photoperiod (12:12 light:dark) temperature-controlled room (10 ± 1 °C). A 7-day period was allowed for the animals to acclimate to their adjusted environment. Behavioural experiments were adapted from those of Tain et al. (2006). After 7 days of acclimatisation, behavioural assays were conducted to test whether geotaxis and phototaxis are altered between uninfected and infected groups. This was achieved by placing specimens in light/dark and vertical choice chambers where every 30 seconds for a 10 minute period the position of the test organism were recorded. Phototaxis of an organism is light-mediated behaviour towards or away from the stimulus: either positive (attracted to light) or negative (attracted to dark). Geotaxis of an organism is gravity-mediated behaviour: either positive (towards gravity i.e. lower regions of the water column) or negative (against gravity i.e. surface of the water column). Phototaxis scoring was measured by issuing a score of 1 in the light side and 0 at the dark every 30 seconds. Therefore a score of 20 was highly photopositive and 0 highly photonegative. The vertical choice chambers were 1000 ml measuring cylinders with dark tiles above and below to produce non-direct uniform light. Geotaxis scores consist of every 30 seconds measuring the height within the measuring cylinder and taking the average over the 10 min period. Therefore high geotaxis score (1000) signifies negative geotaxis behaviour and low geotaxis score (0) is positive geotaxis behaviour.

Figure 23: Phototaxic behavioural assay: Dark and light choice chambers at 10ºC.

Initial studies were conducted by comparing the phototactic and geotactic behaviour of *E. marinus* infected (n = 20) and uninfected (n = 20) with the trematode parasites. As a behaviour effect was observed, *E. marinus* were also exposed to the neurotransmitter serotonin (Sigma). To see whether infected individuals induced a similar effect to those individuals exposed to serotonin, as the manipulation of the serotonin pathway has been linked to some trematode species and other parasite groups (Helluy and Thomas 2003, Tain et al. 2006). *E. marinus* individuals were kept in 100 ml plastic containers using a static renewal system where solutions were changed every 3 days (n=20 for each concentration group). Serotonin solutions were made up to nominal concentrations of 0.01, 0.1, 1, 10 µg/L and a solvent control (0.025% ethanol). Behavioural assays were subsequently performed every 7 days for 3 weeks. Data were compared using non-parametric statistics (either Mann–Whitney or Kruskal–Wallis). Bonferroni corrections were carried out on all pair-wise comparisons of Kruskal–Wallis tests.

3.23 Gene expression study

The behavioural assay findings suggested behaviour modification in infected individuals and consequently the aim of this study was to develop gene biomarkers to elucidate possible neurological mechanisms that may be affected due to the trematode infecting the Langstone Harbour, Portsmouth population. The selection of genes was based on the assumption that the cerebral encysting trematode is modulating the serotonin pathway, although other genes were investigated. Initially, analyses were conducted using flybase (flybase.org) to identify genes involved in the serotonin pathway in *Drospholia*. The sequences retrieved from flybase were then used to perform a local BLAST search against the *E. marinus* transcriptome database to find possible contig matches. Sequences sharing high levels of sequence identifty ($\leq$ e-5) were then taken and an additional BLAST analysis (blastn) was performed against sequences in Genbank (NCBI) to confirm the annotation. Gene candidates were selected on the basis of their direct involvement in the serotonin pathway or within processes that may affect behavioural responses (see Table 4). The only exception to this was a predicted Arginine Kinase gene, which was chosen due to its involvement as a regulating factor of nitric oxide, a specific immune response induced by *M. pabillorobustus*, another behaviour manipulating trematode (Helluy and Thomas, 2010, Ponton et al., 2006). Primers were designed using Primer-3 software (Rozen and Skaletsky, 1999) and synthesised by Eurofins MWG Operon. All PCR reactions had a thermal cycling of 95ºC (4min) followed by 35 cycles of 95ºC (30s), 60ºC (45s), 72ºC (45s) with a final incubation of 5 min (72°C). All reactions had a 25 µl volume containing 1.5 mM $MgCl_2$, 1x buffer, 0.25 mM each dNTP's, 1 unit Taq DNA polymerase, 10ng cDNA (Promega GoTaq®). Using PCR and gel electrophoresis primer concentrations were adjusted, 10mM primer concentration for all primers except *ine* and 5HT1 primers were reduced to 0.5mM (see Figure 24).

Primer	Primer Name	Sequence 5' end to 3'	Target Gene	Genbank ID	Ref. Species	Query Coverage	Max Identity	e-value
1	Ine1F	CGTGGAGGAGCCGTTGCCTG	Neurotransmitter	NM057664.5	*Culex quinquefasciatus*	37%	74%	4.00E-05
	Ine1R	CCTGTGCGGCATCCCTCTGC						
2	Ine2F	CCGGAGGGCAATCTTGCCGG	Inebriated neurotransmitter	NM_001169400.1	*Drosophila melanogaster*	79%	70%	8.00E-09
	Ine2R	CGAGACGAAGCTGGGCCGTC						
3	RHOD1F	CCCGCCAACATGCTGCCTGA	Rhodopsin	DQ85259	*Neomysis americana*	67%	74%	4.00E-74
	RHOD1R	CGGGTGACCGCAGGCTCTTG						
4	RHOD2F	CGCGGTGTCCACCAACCCAT	Rhodopsin	HM044848.1	*Lysiosquillina maculata*	62%	75%	6.00E-98
	RHOD2R	ATTGGCGGGTGATCGCAGGC						
5	ATFF	AACTGGCGATGGCTTGGGCG	Nutrient Amino acid transporter	NM_131991.2	*Drosophila melanogaster*	31%	75%	2.00E-21
	ATFR	CCGAACCATGTGGGATCGGCC						
6	Ty3F	GCGCGGTCTGAAATGCAGCC	Trytophan 5-monooxygenase activation protein	NM_013	*Rattus norvegicus*	82%	71%	2.00E-35
	Ty3R	GCTCCGTTTGGCCGGCTATGA						
7	ADF	TAGCGGAGGCTGCGTCTGGT	Amino acid decarboxylase	XM002403644.1	*Ixodes scapularis*	57%	79%	2.00E-34
	ADR	TATCAATGCGTCCGGGCGGC						
8	PHF	GGTCAAGACCTGGAGCGCGG	Trytophan hydroxylase	AY099427.1	*Aedes aegypti*	49%	72%	6.00E-142
	PHR	GGTGCTGTGGAACACGCGGA						
9	5HT1F	CAACGCAGAGTACGGGGTTGGT	Serotonin receptor 1	NM_057454.3	*Drosophila melanogaster*	No sequence found primers designed directly from sequence		
	5HT1R	GCAAAACGGCGAAATCGAACGGG						
10	AKF	GGAGGCTTAAGCAGTCA	Arginine Kinase	GQ246164.1	*Penaeus monodon*	38%	86%	0.0E
	AKR	GACGGGTTTTTGCCAAAGT						

Table 4: Serotonin drosopholia genes were BLAST searched on the *Echinogammarus marinus* transcriptome database to identify possible serotonin related contigs. These contigs were then selected and a BLAST analysis was performed on the NCBI database to confirm possible function. Primers were then designed using Primer-3 software (Rozen and Skaletsky, 1999) and synthesised by Eurofins MWG Operon

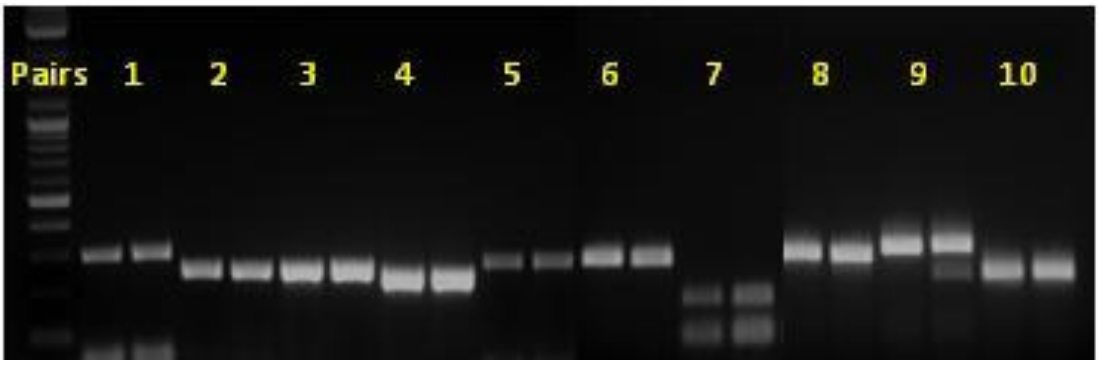

Figure 24: Gel electrophoresis analysis examining the suitability of the primers using pooled control (uninfected) and infected *E. marinus* head cDNA. 1, Inebriated like gene neurotransmitter 1; 2, Inebriated like gene neurotransmitter 2; 3, Putative Rhodopsin gene 1; 4, Putative Rhodopsin gene 2; 5, Putative Serotonin transporter candidate / Amino acid transporter; 6, Putative Tryptophan 5-monooxygenase activation protein; 7, Putative Serotonin receptor – 5HT1; 8, Putative Tryptophan hydroxylase gene; 9, Putative Amino acid decarboxylase gene; 10, Predicted Arginine kinase gene

Six individuals from an uninfected control group and an infected group were dissected as shown in Figure 24. The heads were removed from the body before the first pereon and the antenni were amputated. The six heads were then pooled together and snap frozen using liquid nitrogen, before total RNA was extracted using TRI Reagent® (Ambion) according to manufactures instructions. Briefly, Samples were thawed and left at room temperature for 5 minutes, then centrifuged at 12000g for 10 minutes at 4ºC (ALC, PK121R centrifuge). Supernatant was removed and 600µl of BCP (1-Bromo-3-Chloro-propane) (Sigma) was added to the solution. This was left for 15 minutes at room temperature and samples were then centrifuged at 12000g for 15 minutes at 4ºC. The colourless top layer was removed and placed into a clean microcentrifuge tube, to which 350µl of isopropanol (Sigma, molecular grade) was added. The sample was then vortexed and left at room temperature for 8 minutes, before being centrifuged at 12000g, at 4ºC for 8 minutes and the supernatant removed. The remaining pellet was then washed using 600µl of 75% ethanol (Sigma, molecular grade) and then centrifuged at 7500g, at 4ºC for 5 minutes. The ethanol was removed and the pellet was re-suspended in 50µl molecular grade dH_20 by vortexing gently. The amount of RNA extracted was then quantified using a spectrometer (Thermo Scientific, Nanodrop 1000). #

Figure 25: Female *Echinogammarus marinus*, dashed black lines demonstrating the section of tissue dissected for qPCR analysis. The head was removed before the first pereon and the antenni were amputated.

RNA Clean and Concentrator™-5 columns (Zymo Research Corp, Cat no. R1016) were used to prepare RT-PCR ready RNA, using the manufacturer's guidelines. The RNA was then quantified using a Spectrometer (Thermo Scientific, Nanodrop 1000 Spectrometer), before RNA quality was assessed using gel electrophoresis. From the extracted RNA 250ng was reverse transcribed into cDNA using reverse transcriptase (Promega). Briefly, random hexamers (1µg) were added to the RNA samples, before the samples were heated at 70ºC for 5 minutes, chilled at 4ºC for 5 minutes, spun down and 30µl of transcriptase mix containing DNAse I (3U) with reaction buffer (New England Biolabs) was added (in accordance with manufacturers' guidelines). The samples were then incubated at 25ºC for 5 minutes, 42ºC for 60 minutes and 70ºC for 15 minutes. The resulting cDNA produced was tested to amplify the constitutively expressed GAPDH genes (see Yang et al., 2011 for PCR conditions). To ensure no gDNA remained in the RNA samples, a minus RT control reactions were performed. Real-time PCR using SYBR green based detection was completed using a Real-Time PCR machine (Illumina Eco) and results were generated using ECO software (version 3.0). The cDNA underwent PCR (95ºC for 2minutes, followed by 40 cycles of 95ºC for 15 minutes and 60ºC for 60 seconds, completed with 1 cycle of 60ºC for 95 seconds) with Rox normalisation. For each variant three repeats were completed. The fold-change was calculated using the $\Delta\Delta C_T$ Method (Biosystems, 2008) using GAPDH as the reference gene and the uninfected group as the reference sample.

3.2 Results

3.31 Trematode Identification

The results of the phylogentic analysis based on the 18s, 28S and the ITS regions of the unknown trematode found within *E. marinus* and closely related species revealed all the trematode species in the *E. marinus* populations surveyed belong to the Digenea subclass and the order Plagiorchiida. Inverkeithing trematode belongs to the family Opecoelidae. From the phylogenetic tree based on the 18S we can conclude the trematode species found within the Inverkeithing population are closely related to *Gaevskajatrema halosauropsi* (AJ287514.1) (see Figure 26) with a base pair match of 1878/1902 (99%) across the sequenced region. In addition, the analysis of the 28S sequence, showed *G. halosauropsi* was the closest related (see Figure 27) with a base pair match of 1195/1245 (96%). There was no ITS sequence for *G. halosauropsi* currently available on Genbank, however, the blast analysis on the ITS sequences showed the closest related species to the Inverkeithing trematode were two Opecoelidae spp., an un-described Opecoelidae sp. (AJ241813.1) and *Nicolla Elongata* (AJ241792.1) (see Figure 28), which is found to parasitise the marine fish, *Phycis physis*. From the analysis of the sequences, the trematode infecting the Inverkeithing population can be confidently categorised within the Opecoelidae family.

The Portsmouth and Loch Fleet *E. marinus* populations appears to be infected by the same species or two closely related, with the 18S showing a 99.2% base pair identity. Therefore, no further sequencing was conducted in the trematode found in the Loch Fleet population. The trematode infecting the Portsmouth population appears to belong to the family Microplalloidea on the basis of all rDNA sequence (Figure 26-28). However, phylogenetic analysis reveals no consensus as to which species is most closely related. Generally, the ITS region presents the most variability and is thought to be a good species marker for digeneans. It is highly probable the trematode isolated from *E. marinus* at Portsmouth and Loch fleet is a new species of trematode that belongs to the Microphallus genus.

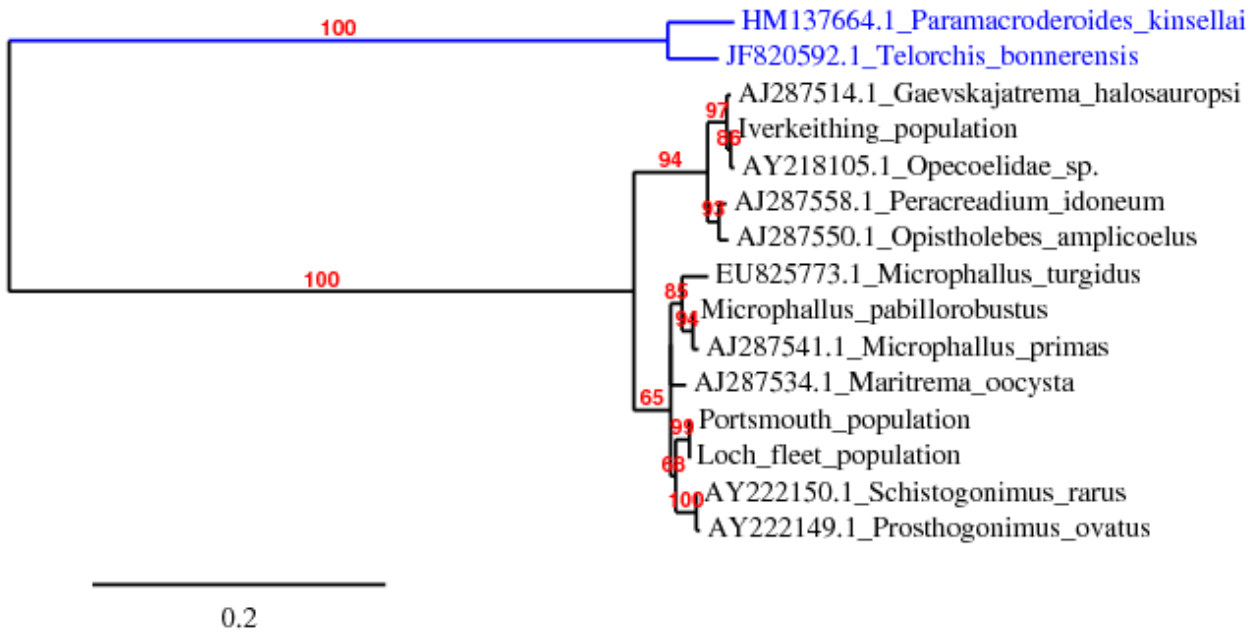

Figure 26: Identification of unknown trematode species infecting *E. marinus* at Portsmouth, Inverkeithing, and Loch Fleet, UK. A phylogenetic tree was generated using available rDNA sequences of digenean trematodes from NCBI and within this study. Sequences were aligned using MUSCLE and a phylogenetic tree was constructed using the maximum likelihood method implemented by the PhyML program. All branches are drawn to scale as indicated by the scale bar representing sequence divergence. Bootstrap values (n = 100) for branches are shown as percentages highlighted in red. The phylogenetic tree was generated as described above using trematode small subunit rDNA sequences (18S); scale bar represents 20% sequence divergence. Outgroups are highlighted in blue.]#

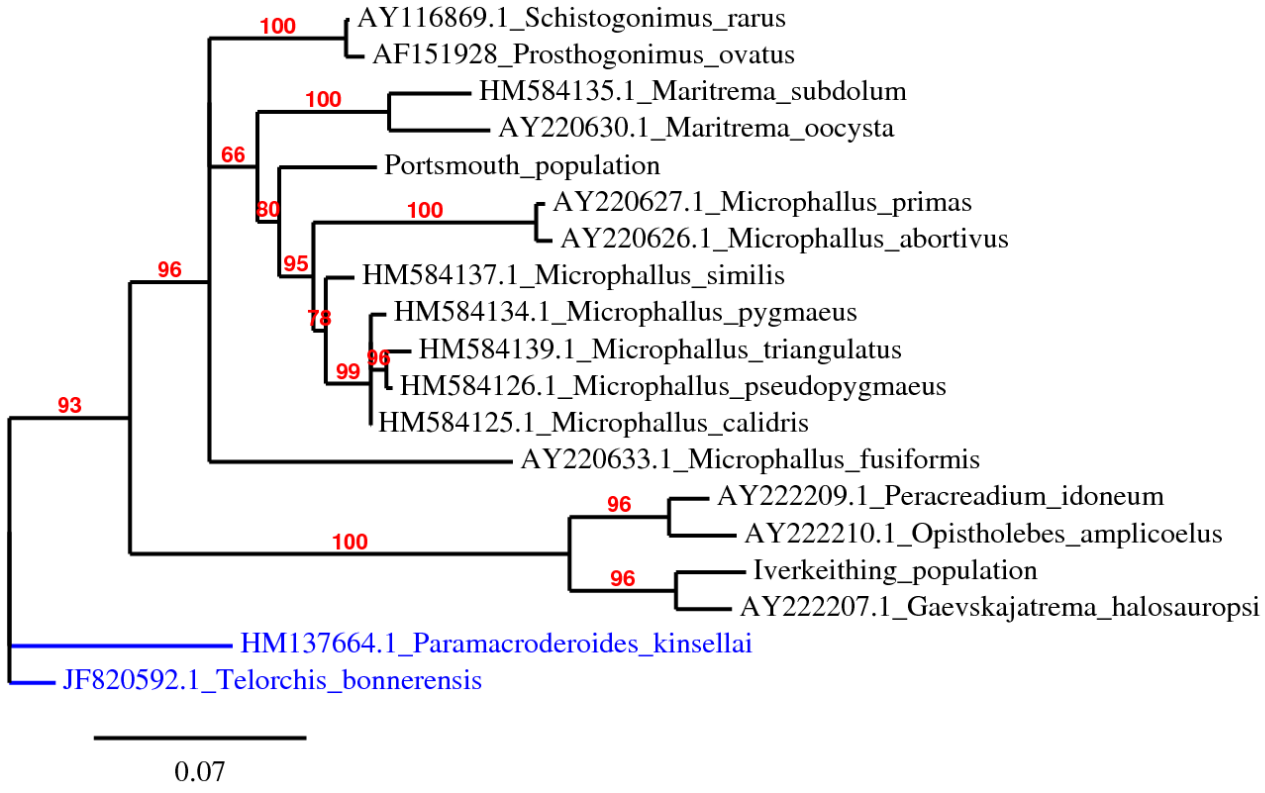

Figure 27: Identification of unknown trematode species infecting *E. marinus* at Portsmouth, and Inverkeithing, UK. A phylogenetic tree was generated using available rDNA sequences of digenean trematodes from NCBI and within this study. Sequences were aligned using MUSCLE and a phylogenetic tree was constructed using the maximum likelihood method implemented by the PhyML program. All branches are drawn to scale as indicated by the scale bar representing sequence divergence. Bootstrap values (n = 100) for branches are shown as percentages highlighted in red. The phylogenetic tree was generated as described above using trematode large subunit rDNA sequences (28S); scale bar represents 7% sequence divergence. Outgroups are highlighted in blue.

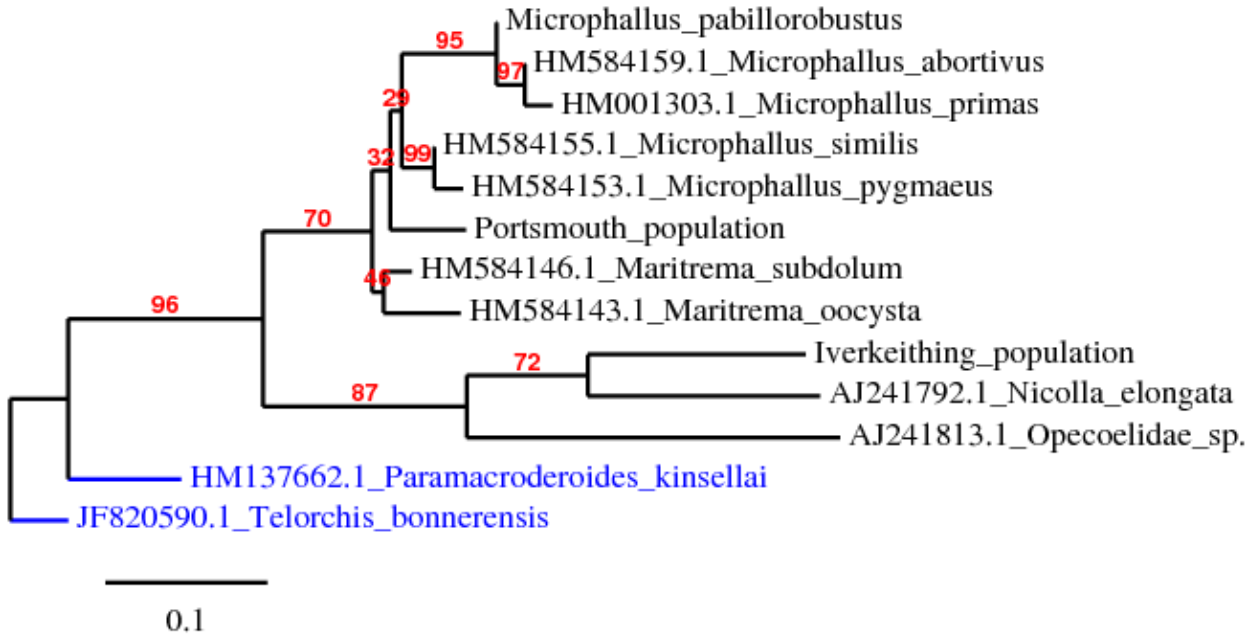

Figure 28: Identification of unknown trematode species infecting *E. marinus* at Portsmouth, and Inverkeithing, UK. A phylogenetic tree was generated using available rDNA sequences of digenean trematodes from NCBI and within this study. Sequences were aligned using MUSCLE and a phylogenetic tree was constructed using the maximum likelihood method implemented by the PhyML program. All branches are drawn to scale as indicated by the scale bar representing sequence divergence. Bootstrap values (n = 100) for branches are shown as percentages highlighted in red. The phylogenetic tree was generated as described above using ITS rDNA region (ITS1, 5.8S and ITS2) sequences, scale bar represents 10% sequence divergence. Outgroups are highlighted in blue.

3.32 Behavioural Study

E. marinus infected with trematode parasites had both significantly higher ($p < 0.001$) phototaxis and geotaxis scores than those of uninfected specimens. Infected specimens spent, on average, approximately 8 times more time in the light and occurred approximately 4 times higher in the water column than uninfected individuals. Phototaxis and geotaxis scores increased with greater concentrations of serotonin for all weeks (1–3) although significant differences were only observed after week 2 ($p = 0.001$) and week 3 ($p = 0.001$) for phototaxis and week 1 ($p = 0.009$) and week 2 ($p = 0.006$) for Geotaxis scores. Mortality was low during all exposure experiments (Table 5), with no mortality within the controls.

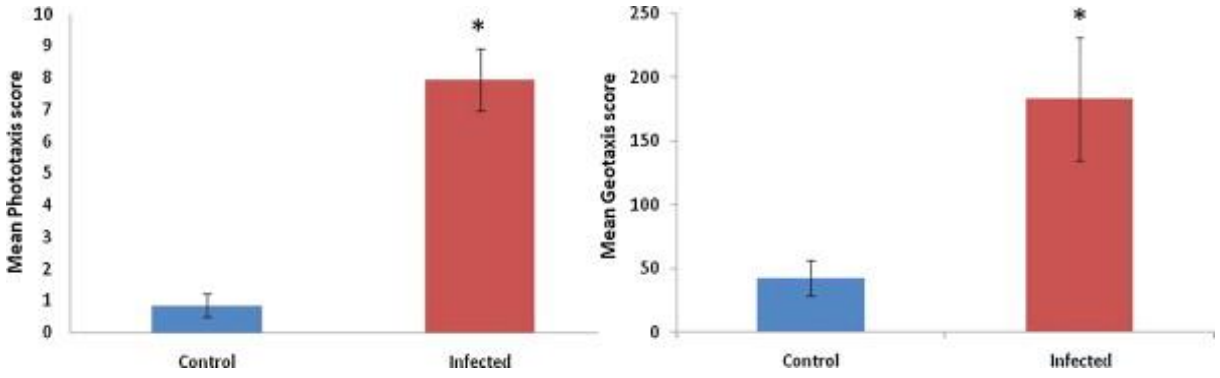

Figure 29: Mean average phototaxis and geotaxis score of *E. marinus* with acanthocephalan infection and a non-infected control group (n = 20 per treatment). Error bars to one standard error. *Significance compared with control ($p < 0.05$).

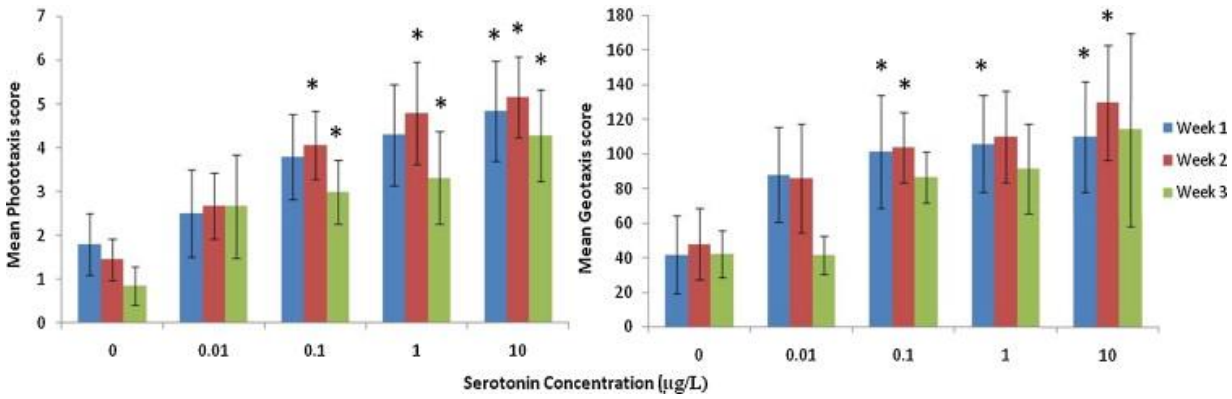

Figure 30: Mean average phototaxis and geotaxis score of *E. marinus* exposed to varied concentrations of serotonin (n = 20 per treatment) over a 3-week period. Error bars to one standard error. *Significance compared with control determined by Mann–Whitney and Bonferroni correction $p < 0.0125$

Behaviour tests	Kruskal-wallis		
	Chi-square	DoF	p-value
Serotonin phototaxis week1	8.507	4	0.075
Serotonin phototaxis week2	17.806	4	0.001*
Serotonin phototaxis week3	19.891	4	0.001*
Serotonin geotaxis week1	13.6	4	0.009*
Serotonin geotaxis week2	14.533	4	0.006*
Serotonin geotaxis week3	5.397	4	0.249

Table 6: Statistical analyses (Kruskal Wallis) of phototaxis and geotaxis responses in male *Echinogammarus marinus* following 7, 14 and 21 days exposure to Serotonin (* = < 0.05). DoF = Degrees of Freedom

Concentration	Mortality
0µg/L	0
0.01µg/L	2
0.1µg/L	4
1µg/L	1
10µg/L	3

Table 5: Total number of mortalities of *E. marinus* per treatment over the 3 weeks serotonin exposure

3.33 Gene expression changes

Analysis of gene expression in infected and uninfected animals indicates several genes that could be influenced by trematode activity (see Figure 31). Results indicate that the putative Tryptophan 5-monooxygenase activation protein gene (Ty3), Putative Rhodopsin gene (Rhod 1 and Rhod 2), and the putative amino acid nutrient transporter gene (AT) are not influenced by the microphallid trematode found in the Langstone Harbour population. The putative serotonin receptor gene (5HT1) shows the highest mean fold change and the putative tryptophan hydroxylase gene (PH) also showed a consistent increased expression in all three trials. The putative Arginine Kinase gene (AK) showed a slight decrease in expression. The inebriated like neurotransmitter genes (Ine 1 and 2) showed a reduced gene expression, with Ine 2 showing a greater reduction than Ine 1 in all three trials. The putative amino acid decarboxylase gene (AD) presented a slight reduction in gene expression overall. Generally, trial 1 showed the greatest alteration in gene expression induced by the trematode infection (see Table 7).

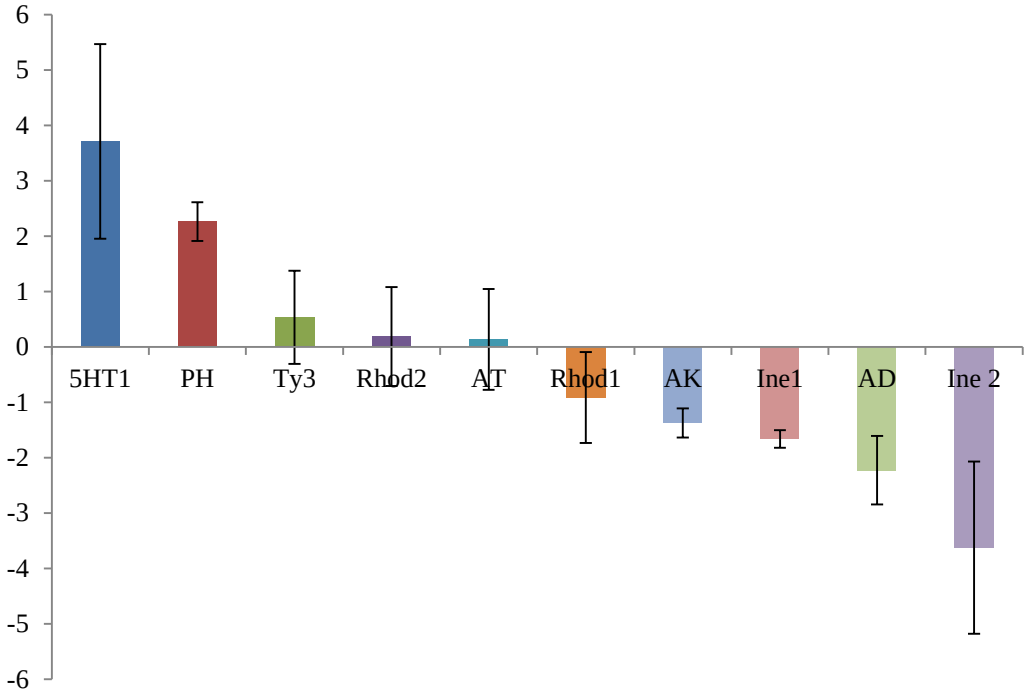

Figure 31: Mean gene expression changes in *E. marinus* induced by trematode infection in three independent trials. qPCR analysis of possible serotonin related genes using *E. marinus* head cDNA pooled (n=6) control (uninfected) and infected trematode for each trial. Bars represent mean fold change and Error bars to one standard error.

Primer	Trial 1	Trial 2	Trial 3	Mean	S.E.
5HT1	7.202	1.645	2.280	3.709	1.756
PH	1.987	2.958	1.845	2.263	0.350
Ty3	1.763	-1.078	0.924	0.536	0.843
Rhod2	-1.577	1.277	0.866	0.189	0.891
AT	-1.469	1.683	0.195	0.136	0.910
Rhod1	-2.233	-1.094	0.590	-0.912	0.820
AK	-1.064	-1.157	-1.895	-1.372	0.263
Ine1	-1.418	-1.611	-1.953	-1.661	0.157
AD	-3.325	-2.163	-1.182	-2.223	0.619
Ine 2	-6.711	-2.410	-1.750	-3.624	1.556

Table 7: Gene expression changes in *E. marinus* induced by trematode infection in three independent trials. qPCR analysis of possible serotonin related genes using *Echinogammarus marinus* head cDNA pooled (n=6) control (uninfected) and infected trematode for each trial. The experiment was repeated three times (trial 1, 2 and 3) to verify findings.

3.4 Discussion

All *E. marinus* populations surveyed were found to be infected by trematodes that belong to the Xiphidiata clade within the Plagiorchiida superorder (see Figure 32). This is not surprising, as there are only two clades within digenea that are common parasites of arthropods, Hemiuroidea and Xiphidiata (Cribb et al., 2003). The Xiphidiate clade displays similar cercarial behaviour among the species, in which cercaria emerges from a gastropod or bivalve and penetrates an arthropod (Stunkard, 1968, Prevot et al., 1976), the second intermediate host. A metacercariae forms within the amphipod and completes its lifecycle when the amphipod is eaten by the vertebrate definitive host, usually fish or bird. This lifecycle is the most ubiquitous within the Digeneans, in terms of abundance among species and prevalence within families.

Inverkeithing trematode belongs to the family Opecoelidae, within the Plagiorchioidea superfamily, with the closest association to *Gaevskajatrema halosauropsi*. In addition, sequences with closest identity to the Inverkeithing trematode were found in marine fish and a marine gastropod, *G. halosauropsi* infects the deep sea fish *Halosauropsis macrochir* (Bray and Campbell, 1996), *N. elongate* infects the marine fish, *Phycis phycis* and the unidentified Opecoelidae sp. (AJ24183.1) infects the sea snail, *Columbella rustica*. Therefore the probable life cycle of the trematode infecting Inverkeithing *E. marinus* population consists of a gastropod as the first host, *E. marinus* as the intermediate host and a fish species as the final host. Whether the Inverkeithing trematode belongs to the Genus *Gaevskajatrema* is debateable, it is indeed very closely related to *G. halosauropsi,* however, other species within that genus, *Gaevskajatrema perezi,* shows reduced association. This discrepancy is most like to be within the grouping of trematode classification and more Opecoelidae sequences on Genbank would elucidate the grouping of this trematode. Although, from the analysis of the sequences, the trematode infecting the Inverkeithing population can be confidently categorised within the Opecoelidae family.

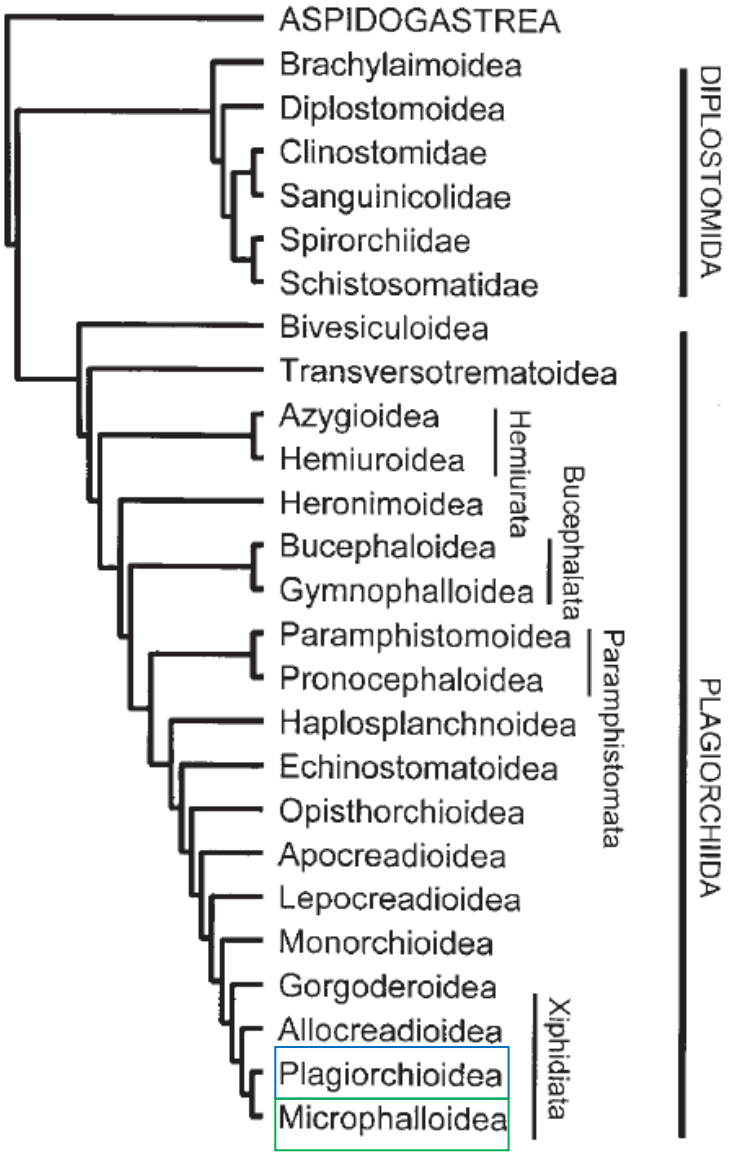

Figure 32: Relationships and higher classification of the superfamilies of the Digenea taken from Cribes et al (2003) analysis. Inverkeithing, Loch Fleet and Langstone harbour (Portsmouth) *E. marinus* population infected by trematode belonging to the Plagiorchioidea (Opecoelidae) and Microplalloidea family, respectively.

The Portsmouth and Loch Fleet *E. marinus* population appears to be infected by the same species or closely related species that belong to one of the most derived taxa within dienean trematodes, the superfamily, Microplalloidea. These sample sites are geographically hundreds of miles apart, yet seemed to be infected by possibly the same species. This could possibly be due to sharing the same final host, which within microphallids is usually a bird or fish. Some bird populations, due to their mobility could theoretically infect geographically distant host populations with the same parasite species. The trematode species infecting the Portsmouth and Loch fleet populations did not reveal a consensus among the phylogentic analysis of which species was closely related. The ITS region has been suggested to offer the best marker for species differentiation (Nolan and Cribb, 2005). Therefore, we conclude the digenean found in *E. marinus* at Loch fleet and Portsmouth population is most likely to belong to the Microphallus genus and is probably a new species. Due to the clear signal reading within the sequencing data, there was no evidence that any of the populations are infected by multiple trematode species. Although, as only 20 infected individuals were pooled to obtain trematode DNA, it is possible there are less prevalent species within the population.

There are many trematode phylogenies based on morphological and molecular data (Cribb et al., 2003, Littlewood et al., 1999, Olson et al., 2001). However, many are incongruent and a general consensus has not been made within the field. Morphological variation is found in the form and positioning of the suckers, the digestive tract and reproductive system. Within sexual adults well defined taxa can often look morphologically highly similar, despite having obvious life cycle stage differences, such as, Allocreadiidae and Opecoelidae, or, Heterophyidae and Microphallidae (Cribb et al., 2003). Interestingly, based on the 18S region *Schistogonimus rarus* and *Prosthogonimus ovatus*, were the closest related to the trematode infecting Portsmouth and Loch fleet populations, these species are believed not to belong to Microphalliodea (Tkach et al., 2003). In addition, *S. rarus* and *P. ovatus*, despite belonging to separate genus, molecularly are well associated and the only morphological character that separates the two species is the position of the male and female genital pores. There is a definite need for trematode

phylogenetics to be revised, improved and combined, as these two methods for understanding phylogeny are not incompatible (Littlewood et al., 1999). This has been attempted with several datasets, the superfamily Microphalloidea, where morphological and molecular data have been revised and combined to gain a better understanding of the phylogentic relationships (Tkach et al., 2003). However, there are still few well resolved clades within trematode that reflect independent datasets (Littlewood et al., 1999).

Significant changes in the phototaxis and geotaxis behaviour was observed in *E. marinus* infected with trematode parasites from the Portsmouth population. Infected specimens spent more time in the light and higher within the water column which is in agreement with past work *M. pabillorbustus* infection of the amphipod *G. insensibilis*, which have also shown to affect phototaxis and geotaxis (Helluy, 1983a, Helluy, 1983b). Several studies have associated parasite infection with changes in the serotonergic activity both through in-situ hybridisation techniques (Helluy and Thomas, 2003) and in-vitro injections of serotonin (Tain et al., 2006, 2007). In this study, phototaxis and geotaxis behaviour increased in a dose-dependent manner following exposure to serotonin from 0.01-10µg/L. Whilst former studies had used $5\mu g/\mu l^{-1}$ Serotonin (Tain et al., 2006, 2007) injected into the heomoceol of amphipods to induce behavioural changes, this study found significant phototaxis and geotaxis responses at 100ng/L within the seawater medium indicating changes could be induced at relatively low concentrations over a chronic emersion period. In conclusion, chronic serotonin exposure mimics the behavioural alterations found in the infected individuals, indicating that this trematode parasite induces some form of adjustment to the serotonin pathway within *E. marinus*.

Perrot-Minnot et al. (2007) studied the predation vulnerability of *G. pulex* infected by the fish acanthocephalan, *Pomphorhynchus tereticollis*, both in laboratory and field conditions. In field studies, the final host predator (Bullhead fish) had 10 times higher proportions of infected *G. pulex* it its gut than uninfected individuals sampled within the same river. In addition, microcosm experiments showed that uninfected amphipods increased the use of refuges in the presence of bullhead predators (Perrot-Minnot et al., 2007). Further study used a Y-maze olfactometer to compare levels of

repulsion to the bullhead in uninfected and infected individuals and demonstrated that gammarids infected by a serotonin modulating parasite are more susceptible to predation than uninfected individuals (Perrot-Minnot et al., 2007). Therefore, increased levels of certain trematode species could have massive impact on population and community structure. The amphipod, *Corophium volutator*, is a second intermediate host to several trematode species that have shown to increase mortality rates in their host under laboratory conditions (Jensen et al., 1998, McCurdy et al., 1999, Meissner and Bick, 1999), in the field this can range from reductions in host abundance to population extinction in relation to trematode infection levels. (Jensen and Mouritsen, 1992, Meissner and Bick, 1997).

Intensity dependent mortality is observed as a result of infection by some microphallid species within the laboratory and field, this can be determined by studying the mean parasite load in the larger size classes, if mean decreases in larger animals it shows heavily infected individuals are removed from the population (Fredensborg et al., 2004). Therefore, a positive linear relationship between *E. marinus* size and the microphallid parasite abundance (Sherhood, 2011, unpublished) suggests the parasite does not induce mortality of host by infection as the parasite can accumulate within the host over time (Dobson and Hudson, 1995). This evidence further supports that the microphallid infecting the *E. marinus* population does not induce a pathogenic effects. In addition, a correlation was found between trematode prevalence and host abundance in Chapter 2. The findings of the behavioural assay and gene expression data strongly suggest specific behavioural alteration capable of increasing the likelihood of transmission.

Serotonin is a monoamine neurotransmitter found ubiquitously among bilateral organisms and is biochemically synthesised from $_{L}$-tryptophan through two short metabolic pathways using two enzymes; tryptophan hydroxylase (TPH) and amino acid decarboxylase (see Figure 33) (Walther and Bader, 2003).

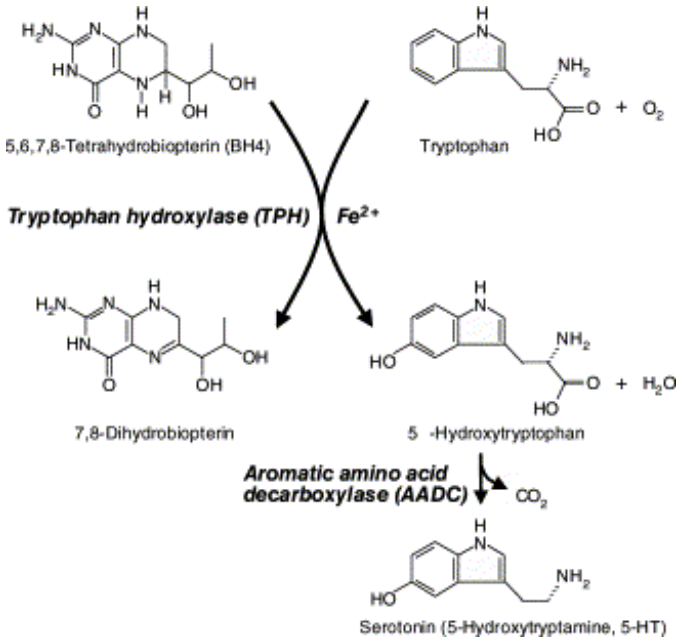

Figure 33: Mechanisms in serotonin synthesis pathway involving tryptophan hydroxylase the rate limiting enzyme and aromatic amino acid decarboxylase. Taken from Walther and Bader (2003).

Within the gene expression study we attempted to link the behaviour alterations observed in the photo and geo-taxic assays with potential neurological gene biomarkers. Several serotonin related genes were highlighted to be affected by the microphallid trematode. The most dramatic fold change in gene expression was found in trial 1 where the putative serotonin receptor 1A like gene showed a 7.2 fold upregulation and the inebriated 2 neurotransmitter like gene presented a 6.7 fold down regulation. The variation observed between the gene expression trials, as well as behavioural assays, can be due to several factors. Parasitology studies have shown that parasite load (Thomas and Poulin, 1998), age and size of parasite (Benesh et al., 2008), age of host (Poulin, 1993), multiple species infections (Cezilly et al., 2000, Haine et al., 2005), seasonality (Brodeur and McNeil, 1989) can all affect manipulation intensity of parasites. In addition, serotonin is involved in many processes and variation in the results was probable. Generally, the trials show similar gene expression patterns, however, increasing the number of individuals used would strengthen findings.

The serotonin $(5\text{-HT})_{1A}$ receptor is an transmembrane, G-protein coupled, somatodendritic autoreceptor within the dorsal raphe nuerons and mediates inhibitory neurotransmission (Hall and Wedel, 1985). The activation of serotonin 1A receptors blocks subsequent serotonin release at the axon terminal, therefore significantly influences serotonin regulation in the brain. Interestingly, the 5HT1A receptor is believed to play a pivotal role in the desensitisation affects following chronic administration of selective serotonin reuptake inhibitor (SSRI) pharmaceuticals in the restraint of 5-HT elevation (Hjorth et al., 2000). The increase expression in this gene could be attempts by the host to counter balance the elevated serotonin levels induced by parasite infection. It would be interesting to investigate the gene expression patterns of the different 5-HT receptor subtypes due to their differential function in modulating serotonin levels.

The inebriated (ine) gene, found in drospholia, is a neurotransmitter (Soehnge et al., 1996). Studies in *Drosophila* found ine gene mutants demonstrate increased excitability of the motor neurons. This gene resembles members of the Na^+/Cl^--

dependent neurotransmitter transporter family, such as serotonin, dopamine, norepinephrine, and catalyses rapid reuptake release of neurotransmitters into the synapse and therefore performs a influential function in neuronal processes (Soehnge et al., 1996). Despite their importance in neuronal function, their role at the molecular level in controlling target neurons is not completely understood. Although, *E. marinus* may not have the ine gene, it is most likely that this is a neurotransmitter of a similar function. Defective reuptake of the substrate neurotransmitter of an transporter can cause overstimulation of motor neurons (Huang et al., 2002) and reduces reuptake of neurotransmitters (Soehnge et al., 1996). The down regulation of this gene could be associated with elevated serotonin levels that could conceivably be associated with the behavioural traits present in infected individuals due to the build up of 5HT1 at the synapse.

Tryptophan hydroxylase is a rate-limiting enzyme that catalyses serotonin biosynthesis in the serotonergic nerves (Hufton et al. 1995). The putative tryptophan hydroxylase gene (PH) was up regulated in the infected *E. marinus* in all trials within the study. The up regulation in this gene, therefore, could have the potential of increasing the biosynthesis of serotonin within the host brain. Studies have shown up-regulation of a tryptophan hydroxylase gene following SSRI drug administration that specifically aims to elevate serotonin levels (Kim et al., 2002, Shishkina et al., 2007). Whether this up regulation is significant enough to induce the behavioural traits shown cannot be certain, however, further study linking serotonin levels found in the brain with the elevated expression of tryptophan hydroxylase would facilitate the validation of these findings. This has been achieved in other parasitology studies via immunocytochemical staining of serotonin levels in uninfected and infected gammarid brains (Helluy and Thomas, 2003, Tain et al., 2007).

Amino acid decarboxylases (AD) are also involved in the synthesis of serotonin and within the study exhibited slight down regulation in all trials with an average fold change of 2.2. This was unexpected, as past work on gammarids infected with behaviour manipulating parasites have shown a higher expression of aromatic L-amino acid decarboxylase proteins (Ponton et al., 2006). Whether the findings within

this study are significant enough to cause serotonin level imbalance is questionable, especially when we are unsure how the parasite induces the behavioural traits observed. It should also be noted that differential expression of aromatic L-amino acid decarboxylase was only shown in gammarid species that display phototaxis alterations (Ponton et al., 2006).

Research in serotonin pathways have been extensively conducted in rats due to pharmacological developments (Ellison, 1977, Steinbusch 1985, Lauder 2006). However, little work has been conducted in marine invertebrates, leaving analysis of results problematic. Gene annotation was assigned from various arthropods sequenced on Genbank (NCBI) (see Table 4) and although the specific function in *E. marinus* cannot be definite, depending on the gene divergence, it is still highly probably that these gene biomarkers have neurologically function. Further study investigating gene function of these possible serotonin candidates would be ideal in piecing together how this parasite manipulates or induces behavioural change on its host. Serotonin level alterations could also result from secretion by the parasite, a response from the host to infection or a specific manipulation of the parasite to the gene pathway. The exact mechanisms that are being alter and how they are being altered cannot be certain, however, behavioural affects appear to be induced by this microphallid species and data suggests that there is neuronal manipulation.

The findings presented here add to the mounting evidence that parasites alter their host's behaviour in ways that promotes transmission. This study has hopefully produced a solid foundation in our understanding of the trematode parasites that infect *E. marinus*, the effects they have upon the host and how they induce these effects.

4. Environmental sex determination in *Echinogammarus marinus*

4.1 Introduction

Environmental sex determination (ESD) is defined as the determination of gender by environmental cues or stimuli during development (Korpelainen, 1990). ESD upholds adaptive control of population sex ratios to promote fitness of an individual at a given time. The adaptive benefit for ESD in most species tends to be size related, as it is an important factor in reproductive success in males and females (Naylor and Adams, 1987). Therefore, ESD mechanisms will favour the sex that benefits most from enhanced growth. In amphipods, increased female fecundity correlates with an increase in size. However, as males guard the females in pre-copulatory behaviour, they need to be larger than the females. To ensure a larger size, a longer period of growth is needed and employing an ESD system, where males are produced earlier in the year, giving a longer period for growth, maximises reproductive success (Watt and Adams, 1994, Naylor et al., 1988a).

The brackish water amphipod, *Gammarus duebeni*, exhibits seasonal sex biased ratios, favouring males in the summer and shifting to a female bias in the autumn (Naylor et al. 1988b). Sex determination can be highly influenced by photoperiod showing responses to light intensity as low as 1 lux (Bulnheim, 1978). At 15^{o}C a long day (>13-14h light) photoperiod regime favours males, whereas, short days (<13-14h light) shifts the ratio to produce more females. However, the ESD response by *G. duebeni* in the laboratory did not correlate with what was occurring in the natural populations (Watt and Adams 1994). This indicated that photoperiod could possibly not be the sole environmental cue accountable for ESD and that a secondary cue was likely to be involved in the sex determination of this species (Watt and Adams, 1993). Temperature was then examined by Dunn et al. (2005) as a potential secondary cue by comparison of four geographically different *G. duebeni* populations. The results showed significant variance between the different

populations demonstrating adaptive variation within the species. Two northern sites had male biased broods under long day warm conditions and female biased broods under short day warm conditions however under cold conditions the sex ratios were reversed. The two southern sites showed no significant affect by temperature, however, produced male biased broods under long day conditions (Dunn et al., 2005). Other evidence of dual ESD comes from the fish species, *Poecilia sphenops*, that uses salinity and temperature as environmental cues (Barón et al., 2002). Utilising photoperiod and temperature as environmental cues can allow for greater accuracy in timing, ensuring gender specific production during its most suitable period for male and female growth (Dunn et al., 2005). The study also demonstrates the degree of ESD varies among populations geographically. This could be due to the fact that some temperature and photoperiod combinations do not occur in the environments of some populations during their breeding season and they have thus have adapted to different ESD conditions. In conclusion, Dunn et al (2005) demonstrated an interaction of temperature and day length as cues for ESD as well as variation in the degrees of ESD between the different populations, thus demonstrating adaptive variation within the species.

It is well-documented that *Echinogammarus marinus* presents a range of sexual phenotypes (Ford et al. 2005). Intersexuality and female bias has been linked with vertically transmitting parasites that are believed to feminise male embryo hosts as a reproductive strategy to facilitate parasite transmission to the next generation (Kelly et al., 2004, Yang et al., 2011). However, other factors known to influence crustacean sex determination pathways, such as ESD, have yet to be explored in *E. marinus*. Past studies have reported *E. marinus* populations showing temporal and geographical variability in their sex ratios (Martins et al., 2009, Vlasblom, 1969., Yang et al., 2011), however, no current published data has shown whether ESD is present within this species. To address this issue, this study investigated the effect of photoperiod on the sex ratio of broods in *E. marinus*. In addition, these results were compared with sex ratios from a two-year field study from a population of *E. marinus* at Langstone Harbour, Portsmouth, a population uninfected by known feminising parasites (Yang et al., 2011).

4.2 Methods

4.21 Laboratory study

Animals were collected from Langstone harbour, situated in Portsmouth, southern England, U.K. site (50º47’23.13N 1º02’37.25W) and a total of 60 pre-copular pairs were placed in 150-ml pots that contained filtered seawater with fucoid seaweed (*Ascophyllum nodosum*) for food. Both food and seawater were changed approximately every 4-5 days. In other gammarid species, ESD is influenced 3-4 weeks after release from the mother’s brood pouch (Bulnheim, 1978, Naylor et al., 1988b). However, no assumption was made at what point environmental cues might influence sex determination (either as a zygote or juvenile stage). Therefore pre-copular pairs were assigned at random to one of the photoperiod regimes: 16 hours light and 8 hours dark or 8 hours light and 16 hours dark (30 broods per light regime at 15^{o}C) to mimic the extremes of long day and short day conditions in the field. This ensured that the broods were exposed to the chosen photoperiod at all developmental stages, although confounded maternal effects cannot be ruled out. Once juveniles left the female brood pouch (approximately 30 days after egg release) all adults were removed to avoid cannibalism. Despite the microsporidia infecting this population, *D. berillonum*, being a non feminiser, all brooding females were screened for microsporidian parasites using PCR (Yang et al., 2011) and broods were eliminated if the mother tested positive (n = 11/60). *E. marinus* sex ratios were determined when sex could be distinguished morphologically after approximately five months.

4.22 Field Study

To assess sex ratios in the field, *E. marinus* were collected over a two year period between December 2009 to December 2011 from Langstone Harbour (Portsmouth, UK). Hours of daylight at this latitude vary between approximately 8-16 hours throughout the year. Samples were taken by selecting five $1m^2$ quadrats (total area = $5m^2$) in the intertidal zone during low tide. All algae and surface sediment (approximately 2cm in depth) was retrieved and stored in polythene bags. In the laboratory, samples were washed and decanted through a 0.7 mm sieve and all algae were scraped to ensure no individuals were left. All amphipods were collected and stored in 70% ethanol where *E. marinus* specimens were separated into males, females and juveniles. Generally sex could be determined within individuals that were approximately over 10mm in length. *E. marinus* males were distinguished by the presence of enlarged gnathopods and genital papillae, whereas, females were distinguished by much smaller gnathopods and oostegites (brood plates). Individuals not presenting any of these features were grouped as juveniles.

4.3 Results

4.31 Laboratory study

A significant difference in the sex ratios between the two light regimes' was observed (Mann-Whitney test; $p<0.001$) in the laboratory breeding experiments with a male bias recorded over a long day photo regime (mean = 61.5$\pm$0.84% male broods; n = 16) and a female biased over a short day photoperiod regime (mean = 43.5$\pm$0.94% male broods; n = 12). In addition, using negative binomial regression a significant relationship was shown in sex ratio proportions comparing the long and short light regimes (Wald = 18.607; DF = 1; $p<0.001$). No significant difference in the mean number of juveniles produced per female was observed for short and long day regimes, (27.5 $\pm$ 0.9 and 26.6 $\pm$ 0.8 respectively), when normalised to size (ANCOVA; P = 0.0476, F = 0.522, DF = 1). Furthermore, there was no significant difference (Chi-square X^2 = 0.02; DF = 1 ; P = 0. 823) in the brood survival between the long (67%) and short day regimes (69%).

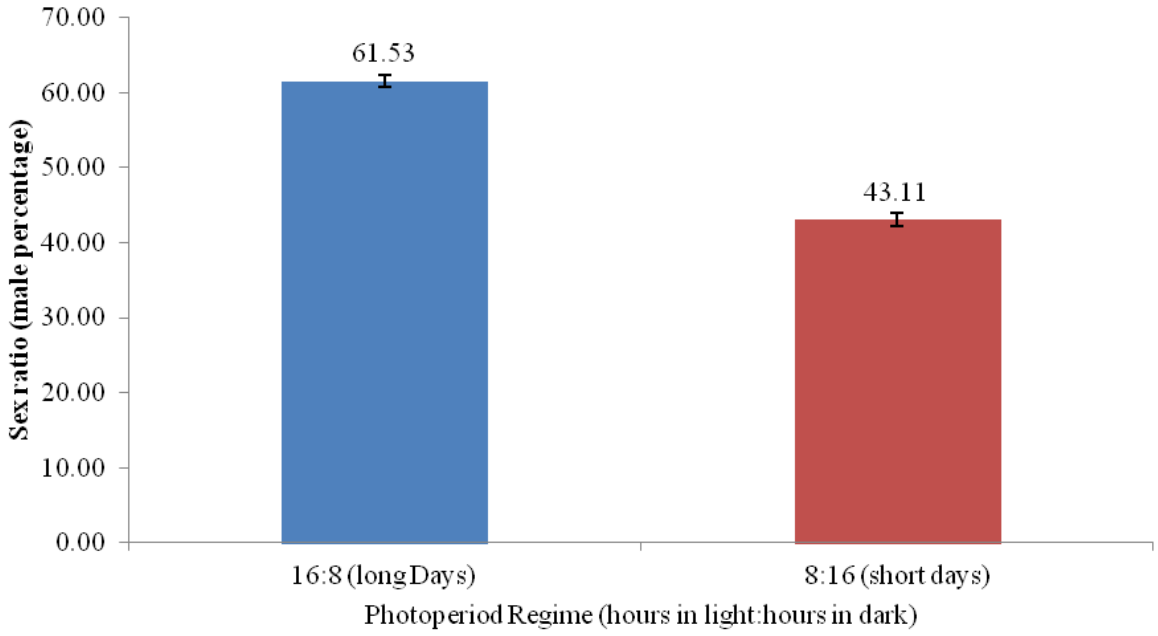

Figure 34: Sex ratio of *Echininogammarus marinus* broods from Langstone harbour (Portsmouth, UK) under different day lengths conditions and kept at 15±1°C. 16 hour light regimes (n=16) and 8 hour light regimes (n=12). Error bars to one standard error.

4.32 Field Study

During the two year field study 1810 adult *E. marinus* were collected and sexed, of which 910 were males and 900 were female. Examination of the monthly sex ratios revealed a general male bias over the late summer and early winter months (Aug-Dec) and a female bias during late winter and early summer months (Jan-Jul) for both years (Figure 35). Average day length hours and temperature were plotted against sex ratios to determine any relationship. In addition, environmental parameters were offset by four months forward to allow for the ~4 month developmental period between when the eggs were fertilised and the point at which the sex could first be determined (Figure 35). The monthly sex ratios were statistically analysed against the daylight hours and environmental parameters (+4 months) using multiple linear regression and photoperiod proved to be significant ($P < 0.001$, $R = 0.564$, df = 2, F = 15.704; Figure 37) indicating that photoperiod correlates with sex ratios in the field ($P = 0.044$; t = 2.017; Beta = 0.113). However, temperature failed to show any correlation ($P = 7.25$; t = 0.352; Beta = 0.020) (see Figure 36). When directly comparing the field data with the laboratory findings the brood sex ratios correlate and fit within confidence bands associated with the field data (see Figure 37). Multicollinearity analysis was conducted and indicated a low association in temporal monthly samples (Tolerance = 0.846; VIF = 1.182). However, temperature showed high collinearity with sex (Tolerance = 0.115; VIF = 6.434) and therefore a possible interaction between temperature, photoperiod and sex cannot be ruled out. No presence of temporal autocorrelation was observed (Durbin-Watson = 1.828) indicating independence of temporal data.

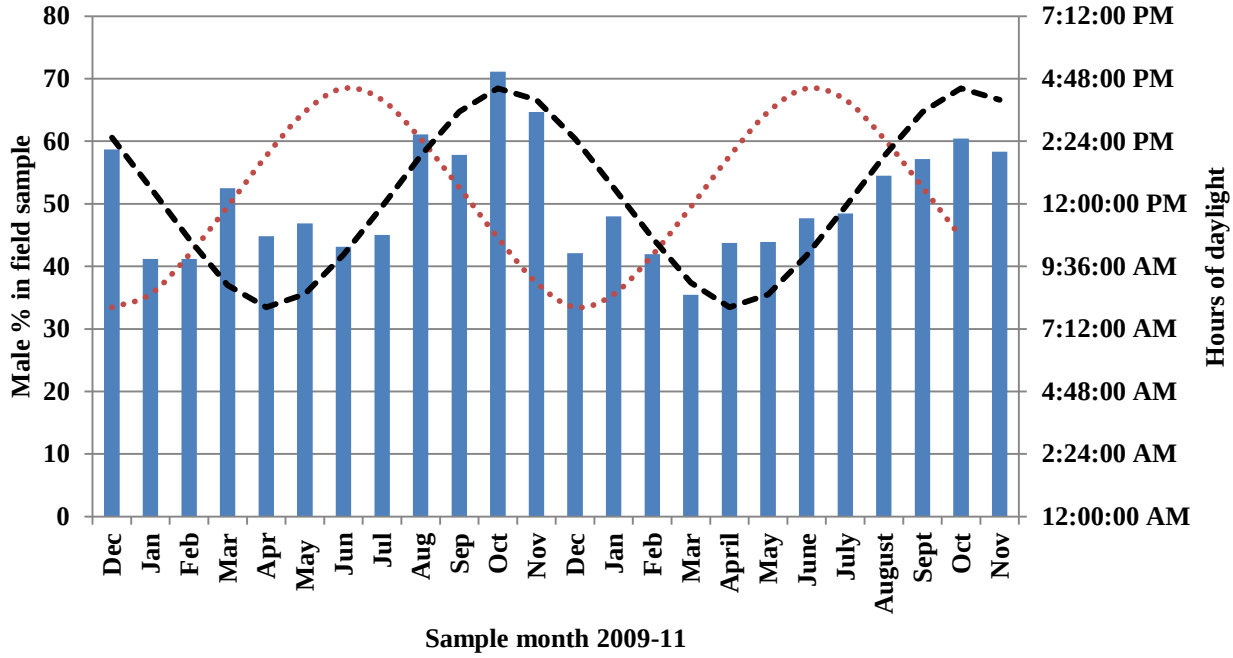

Figure 35: Sex ratio of *Echinogammarus marinus* adults from Langstone harbour (Portsmouth, UK) collected between December 2009 and November 2011. Red dotted line represents monthly average hours of day light. Black dashed line represents monthly average hours of day light (+ 4 months)

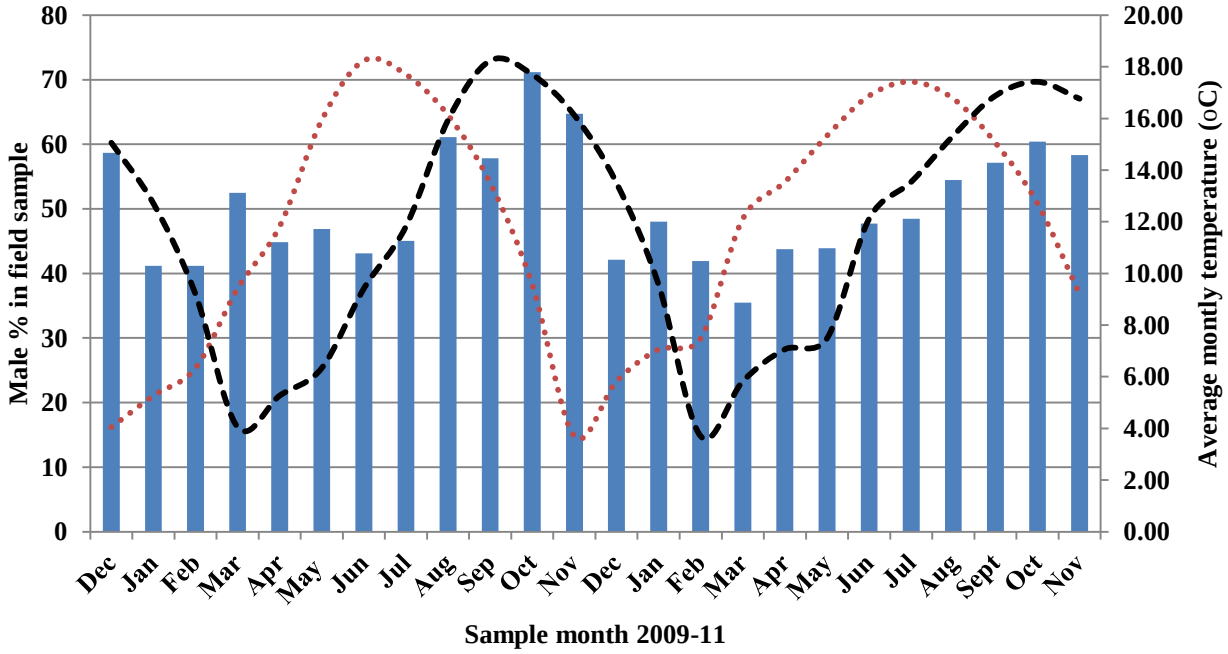

Figure 36: Sex ratio of *Echinogammarus marinus* adults from Langstone harbour (Portsmouth, UK) collected between December 2009 and November 2011. Red dotted line represents monthly average temperatures. Black dashed line represents monthly average temperatures (+ 4 months).

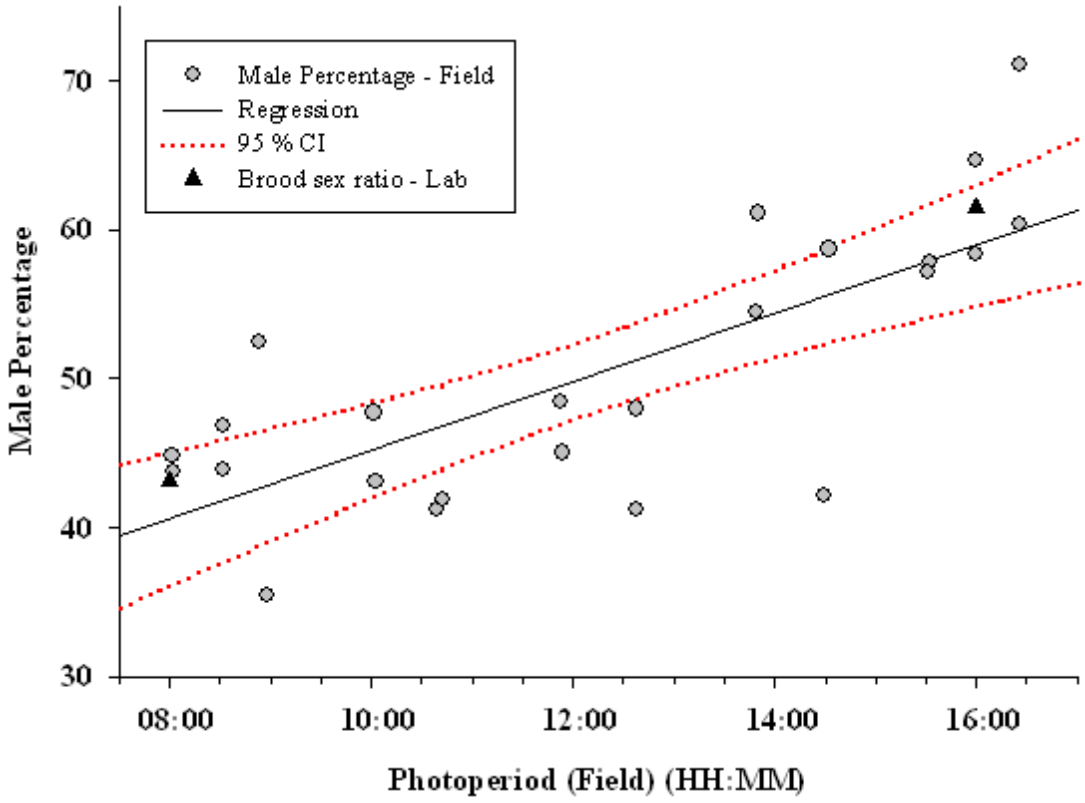

Figure 37: Linear relationship between photoperiod and percentage male *Echinogammarus marinus* from Langstone Harbour, Portsmouth (UK). Field data 2009-2011 (circles) and laboratory data (triangles).

4.4 Discussion

Sex determination and other reproductive processes still remain largely unknown in many aquatic invertebrate species. Amphipods are extensively used in ecotoxicology studies, with an increasing emphasis on reproductive endpoints (Hyne, 2011). However, to truly understand whether anthropogenic influence is currently an issue, it is critical that all the mechanisms governing reproductive processes are fully evaluated. Ecologists have criticised the lack of basic knowledge in the biology of well-studied fauna, as well as highlighted an extreme bias in our knowledge towards vertebrates (Tyler et al., 2012). Environmental conditions can be influential factors in reproduction and development, and a better understanding of how these altered conditions affect reproductive biology in organisms is required. Such an understanding will be crucial in our ability to model and predict population levels in a changeable environment (Visser et al., 2004).

The aim of this study was to demonstrate whether *E. marinus* displays ESD under laboratory conditions and whether this correlates with sex ratios detected in the field. Photoperiod was shown to be an influential factor in sex determination and a significant correlation was observed between sex ratios detected in the laboratory and field. Despite this, the regression model only accounted for 56% of the variation in the data set, which suggests that other environmental factors (e.g. temperature) and inter-individual variation may also be involved. The range of sex ratios from the field study displayed large variation, with extremes of 36% males (Mar 2011) and 71% males (Oct 2010), strongly suggesting adaptive sex ratio variation over the 1:1 Mendelian sex ratio. These swings in sex ratio were mirrored over the two-year study, with the October months possessing the highest proportions of males and January through to March having the lowest. Interestingly, when comparing the total number of males and females over the two-year study, the Portsmouth population displayed an overall 1:1 sex ratio (50.3% males). So, while the two-year field study does highlight a seasonal gender bias, the population overall does not produce more of one gender over the course of two years. The laboratory experiments resulted in a male bias over long day and female bias over the short day photoperiod. These findings are consistent with those of the estuarine temperate amphipod species,

Gammarus duebeni, that showed females (taken from in southern sites within the UK) produced male biased broods under long day laboratory conditions (Dunn et al., 2005). Although laboratory and field data correlate well and support photoperiod as an ESD cue, a second cue (e.g. temperature) for ESD has been inferred in other aquatic species (Dunn et al., 2005, Baron et al., 2002) and should also be considered for *E. marinus* due to high collinearity between sex and temperature indicating a possible interation. In addition, due to experimental constraints, a consistent temperature was required for both photo regimes to identify whether photoperiod was an influencing factor. However, in the field under short day regimes (8hrs of light) the population would normally endure between 4.05-5.82^{o}C. Therefore, it should be noted that the population would not normally encounter these conditions and is biologically unrealistic.

The *E. marinus* population used in this study has a continuous reproductive output and breeds throughout the year, producing male and female bias seasonally. This is despite the prediction that if a breeding season is unrestricted and there is a generations overlap, ESD is no longer advantageous and will revert to a genetic system where males and females are produced simultaneously (Naylor et al., 1988b). Given the apparent costs of using ESD, such as intersexuality and inconsistent environmental conditions, the benefits for this population are not obvious. This could suggest that ESD in this population is ancestral and, whether advantageous or not, has been retained.

The *E. marinus* population used in this study enabled the reliable detection of ESD because it is uninfluenced by known feminising parasites (Yang et al 2011). However, the variable presence of such parasites makes direct comparisons of sex ratios between populations difficult. Among *E. marinus* populations so far studied, female bias is common. Vlasbloom (1969) observed female bias in *E. marinus* populations from the Netherlands, with an approximate average of 40% males. In addition, although a female bias was mainly present, sex ratio fluctuations appeared similar to those seen in this study were observed but with male increase occurring two months earlier. In comparison, southern latitudinal populations (Mondego

estuary, Portugal) have shown female bias in autumn and winter, with a reversal in the spring and summer months. However, further south in the estuary, male bias was observed during winter, with female bias occurring during part of the summer and autumn (Maranhao et al., 2001), a result similar to that found in this study. The cause of this variation is unknown but could be due to environmental conditions specific to the latitudinal positions, as well as other sex determining factors, such as the already mentioned feminising parasites. Within the UK, well-documented *E. marinus* populations have high female bias that is clearly correlated to parasite infection (Ford et al., 2006; Short et al., 2012b), and although these populations also present fluctuating sex ratios, it is problematic to separate the influence of photoperiod and parasites. Although it is clear that *E. marinus* sex ratios can vary considerably, reasonable comparisons of environmentally induced sex ratio fluctuations in *E. marinus* will require detailed surveys of other populations not influenced by feminising parasites.

Within certain crustacean species environmental parameters can be key variables in sex determination. In spite of research into the ways that these cues affect sex ratios, the precise way these cues act upon and manipulate gender, as well as, the genetic control of ESD is largely unknown. In addition to being linked to parasite infection, amphipod intersexuality has been associated with populations that possess ESD (Dunn et al., 1996). *E. marinus* populations consistently present a small fraction of intersex individuals that are not infected with feminising parasites (Yang et al., 2011). Indeed, the population used for this study has no known parasitic feminiser, yet presents notable levels of intersex (Yang et al., 2011). Populations that possess high levels of ESD have been linked with a high frequency of intersexuality. It has been suggested that this is caused by ESD occurring post conception. Therefore, the delayed genetic control of sexual development being the cause of individuals not fully sexually differentiating (intersex) (Dunn et al., 1990). However, at which developmental stage ESD occurs and how the cues act upon the sex differentiation processes is largely unexplored in most species. Alternatively, the non-parasite induced intersex phenotypes could be the result of an underlying ESD mechanism that has been disrupted due to intermediate environmental signals (Dunn et al., 1993). The mechanism of ESD could also increase the susceptibility of the host to

parasite-induced feminisation. Feminising parasites have been shown to have higher prevalence in populations with high levels of ESD. It has been suggested that the delay in sex determination resulting from the ESD pathway causes host vulnerability to the manipulation by feminising parasites, as the parasites are more easily able to override an ESD pathway than they are a genetic based system (Dunn et al., 1995).

E. marinus has a large geographical range from approximately 39^{o}N, where average daylight hours can range from 9-15hrs throughout the year, up to 65 ^{o}N, where 24 hours of daylight occur at certain times of the year. This is important when considering the potential implications of climate change. There is evidence suggesting that species distribution shifts occur as a result of a changing climate (Parmesan and Yohe, 2003). Species that have a sex determination pathway influenced by photoperiod and more prominently temperature may well be latitudinally constrained. As a result, *E. marinus* populations may be forced to adapt to increased temperatures or altered photoperiods. Given the ecological importance of the species, a better knowledge of sex determining factors, in particular ESD, will be required if we are to fully understand the impact of a changing environment.

5. Parasite prevalence and transmission in *Echinogammarus marinus*

5.1 Introduction

The study of sex distorting parasites is of great importance due to the impact they can have upon population dynamics and the selective pressures they exert on the evolution of host sex determining mechanisms. Female biased *Echinogammarus marinus* populations have previously been linked with infection by microsporidia (Yang et al., 2011). However, other parasites groups known to influence crustacean sex determination or differentiation, such as *Wolbachia* and Paramyxea, have yet to be fully explored. Therefore, identification of these parasite groups in female biased populations and investigations into their mechanisms of transmission will hopefully elucidate how parasites might be influencing *E. marinus* population dynamics.

The bacterium, *Wolbachia*, is a well documented feminising parasite found to infect terrestrial isopods (Bouchon et al., 1998). In one such isopod, *Armadillidium vulgare*, the bacteria can convert genetic males into neo females (Legrand and Juchault, 1970). It was initially thought that *Wolbachia* was restricted to the isopod group within crustaceans (Bouchon et al., 1998). However, closely related *Wolbachia* strains have now been identified in amphipods, ostracods and cirripeds suggesting their prevalence in non-isopod crustaceans has been underestimated (Baltanas et al., 2007, Cordaux et al., 2001, 2012). To date, three marine amphipod species (*Orchestia gammarella, Talitrus saltator* and *Talorchestia deshayesii)* has shown *Wolbachia* infections (Cordaux et al., 2001, 2012). However, studies have not linked *Wolbachia* infection as a feminising agent within amphipods.

Microsporidia are obligate eukaryotic intracellular parasites that are widespread and infect a diverse range of vertebrates and invertebrates (Wittner, 1999). In amphipod

populations, microsporidia infections appear ubiquitous. Terry et al., (2004) screened 12 amphipod species and found all species exhibited infection and a total of 16 different microsporidia species were detected. The high occurrence and diversity of these parasites suggests they are well adapted to utilising amphipods as hosts. In addition, the majority of amphipod groups studied had female biased populations and female bias within their infection rates indicating sex ratio distortion (Terry et al., 2004). The amphipod, *Gammarus duebeni* has been reported to be a host to at least four fully feminising microsporidia (Bulnheim, 1978, Dunn et al., 1993, Terry et al., 1999). *Nosema granulosis* has been observed to infect up to 46% of females in several natural *G. duebeni* populations (Terry et al., 1998). Breeding experiments have demonstrated *N. granulosis* possessing feminising effects with 86% of the offspring of infected mothers developing as females (Ironside et al., 2003). Level of infection within *G. duebeni* populations is closely associated with parasite burden and efficiency of transmission (Dunn and Hatcher, 1997b). This can vary among populations and burden is thought to be related to what tissue is initially targeted e.g. gonadal tissue (Dunn et al., 1995). Dunn et al. (2006) studied transmission efficiency of two microsporidia *N. granulosis* and *D. duebenum* on the host *G. duebeni*. *N. granulosis* was reported to vertically transmit up to 82% of the host brood and *D. duebenum* transmitted up to 72% of the host brood. Both species showed high transmission, however, differed in their replication. As host developed, burden increased in *N. granulosis* and decreased in *D. duebenum* (Dunn et al., 2006). These microsporidian studies demonstrate the great influence that these parasites can have over amphipod sex determining mechanisms. Within *E. marinus* there is still a lack of knowledge regarding microsporidia transmission and their effects. Understanding transmission (number of infected eggs in host brood) in *E. marinus* will hopefully reveal reproductive strategies and affects these parasites could possible induce.

Paramyxeans are parasitic protists that are known for causing sexual dysfunction and mass mortalities within molluscs (Villalba et al., 1993). In the amphipod, *O. gammarella* a correlation between paramyxean infection and female biased broods, as well as intersexuality has been observed (Ginsburger-Vogel, 1991, Ginsburger-Vogel and Desportes, 1979). Paramyxid taxonomy is still open to debate,

parasitologists have often placed this group in the phylum Cercozoa (Cavalier-Smith and Chao, 2003). Although, a phylogenetic anaylsis based on molecular data suggests that this is incorrect and indicated that the Paramyxeans belong to a separate phyla (Freeman, 2009). The phylum Paramyxea consists of three genera: *Marteilia, Paramarteilia,* and *Paramyxa* in which species have been grouped in accordance with their life cycle (Feist et al., 2009). Paramyxeans have an unusual cell division in which it undergoes a series of internal cleavages where the daughter cells are found within the mother (stem) cell and has only been observed to infect crustacean and mollusc tissue (Audemard et al., 2002, Feist et al., 2009, Ginsburger-Vogel and Desportes, 1979). Although, limited work has been conducted on Paramyxea within amphipod hosts these marine parasites have the potential to manipulate sex determining mechanisms within *E. marinus* and findings would provide some insight in this relatively new area of study.

Transmission efficiency and artificial infection experiments have not yet been explored in *E. marinus*. The aim for this study was to investigate the role of parasite sex determination (PSD) using a population (Inverkeithing, Scotland) known to have female biased sex ratios and high intersex numbers. The Inverkeithing, *E. marinus* population has extreme female bias which has previously been linked with microsporidia parasites (Ford et al., 2006, 2007) and recently an un-described Paramyxean closely related to *Marteilia refringens* and *Marteilioides chungmuensis* was identified within the population (Short et al., 2012a). Identification of further parasite species that have the potential to influence crustacean sex determination will be achieved by conducting a screen of known sex distorting parasites that infect amphipod species (microsporidia, *Wolbachia* and Paramyxea). Prevalence rates will be conducted within the population sexual phenotypes to indicate any sexual bias with infection. Furthermore, a verification of whether the parasites are vertically transmitted and their transmission efficiency (proportion of eggs from brood infected) will be conducted. Artificial infection experiments will be attempted to see whether vertically transmitting parasites can horizontally transmit within *E. marinus*.

To our knowledge the *E. marinus* Langstone harbour, Portsmouth population has no feminising parasites. In chapter two, we established that there are low levels of

intersex, no overall female bias and no evidence of sex distorting parasites within the population. The only microsporidia species present is *Dictyocoela berillonum*, which presents infection patterns inconsistent with possessing any sex distorting effects (Terry et al., 2004). Therefore, in the artificial infection experiment individuals from the Portsmouth population were horizontally infected with possible sex distorting parasites contained in the tissue of infected individuals from the Inverkeithing population. This will ensure the test population were not already infected with any sex distorting parasites prior to the experiment. Verification of whether *D. berillonum* vertically transmits and examine the parasites transmission efficiency to compare microsporidia species in a female and non female bias population will also be conducted.

5.2 Materials and Methods

5.21 Parasite Identification

E. marinus were collected from beneath seaweed and rocks in the intertidal zone during low tide in Inverkeithing Scotland (56°1'38"N 3°23'37"W) in March 2012 (see Figure 21). The parasite screening of Microsporidia, Wolbachia and Paramyxea in the Inverkeithing population was conducted as in chapter two (as below section 2.22). Animals were anaesthetised in clove oil (0.4µl/ml) and the sexual phenotype was determined. DNA from each individual was extracted using the DNAeasy kit (Qiagen, UK) following the manufacturers protocol. 40 males and 64 females were pooled; this pool was subsequently screened for Microsporidia, Paramyxea and *Wolbachia* (see chapter two for PCR conditions). Once the parasite groups infecting the population were identified individual screenings were conducted to determine infection rates within the various sexual phenotypes.

5.22 Transmission

Ovigerous females from the Langstone Harbour Portsmouth population that were infected by *D. berillonum* and Inverkeithing females that showed infection with any potentially sex distorting parasites had their broods removed and DNA was extracted using DNAeasy kit (Qiagen, UK) following the manufacturers protocol. The pooled brood DNA enabled verification of whether the parasites present were vertically transmitting (see chapter two for PCR conditions). *E. marinus* broods were selected to represent each parasite group that showed VT and an uninfected control and the DNA of each egg or embryo was extracted individually, as described above but with an additional 10 minute RNAse step. The broods then were screened for the presence of the parasite groups to establish their transmission efficiency (see chapter two for PCR conditions). In addition, sex ratio data of broods taken from *D. berillonum* infected mothers which was conducted in chapter four will be analysed and presented (see section 4.2 for methodologies).

5.23 Artificial infection

Following anaesthetising in clove oil (0.4µl/ml), the specimens from Inverkeithing had their head, gut, and hepatopancrease removed. The body tissue was cut laterally in halve. One half was stored in a 1.5 ml ependorf containing 1 ml of seawater at 4^{o}C. The gonadal and muscle tissue in the other half was used for DNA extraction using the Phire® Animal Tissue Direct PCR Kit (using the manufacturer's guidelines) in which the DNA extracted from the muscle and gonadal tissue was incubated (in 20µl of dilution buffer and 0.5 µl DNA release additive) for 5 minutes at room temperature and then 2 minutes at 98^{o}C. The solution can then directly go into the PCR reaction and was used to screen the samples for parasite infection (as described previously). This method reduced time in DNA extraction and PCR screen, so that the tissue stored in seawater is as fresh as possible and reduces the likelihood of parasite mortality for subsequent artificial infection.

The Inverkeithing animals that screened positive for vertically transmitting parasites were used to artificially infect the Portsmouth sample population either by feeding or infecting. The fed group were starved for 7 days and then the infected tissue was placed in the tank. The injected group was inoculated using a Hamilton® Syringe, 700 Series, Removable Needle (Sigma). Muscle and gonadal tissue was homogenised and injected between the 4th and 5th pereon of the amphipods. Animals in the control groups were fed and injected tissue from the Portsmouth *E. marinus* population to ensure no feminising parasites were infected in the control groups. Ten males and ten females from the Portsmouth *E. marinus* population were infected per group and were previously checked to ensure no intersex individuals were used. After four months, the animals that had survived were anesthetised in clove oil (0.4µl/ml), checked for any signs of intersexuality and then had muscle and gonadal tissue dissected and DNA was extracted using the DNeasy kit (Qiagen, UK) following the manufacturers protocol and subsequently tested for infection.

5.3 Results

5.31 Parasite Identification

PCR analysis from Inverkeithing (Scotland) revealed an unidentified paramyxean and the microsporidian, *Dictyocoela duebenum*, to be infecting the population. No Wolbachia infection or any other species of microsporidia or Paramyxea was present. In total, 64 females and 27 males were individually tested for the paramyxean and *D. duebenum* (see Table 8). Overall, 59.6% of the sample population was uninfected, 26.0% presented co-infection, whereas the paramyxean and *D. duebenum* only infections were 12.5% and 1.9%, respectively. Overall infection prevalence of *D. duebenum* (28%) and paramyxean (39%) showed no significant difference between them (X^2 = 2.6241 ; df = 1 ; P = 0.1053). Co-prevalence was high with 68% of the paramyxean infected population presenting co-infection with *D. duebenum* and 93% of the *D. duebenum* infected population presenting co-infection with the paramyxean. Statistical analysis indicated that these two parasite groups have an significant association to each other that this co-infection prevalence is greater than expected (X^2 = 7.5591 ; df = 1 ; P = 0.0060). Infection rates were female biased, with 50% of all females presenting infection by at least one of the parasites, whereas, within the male population only 25% showed infection. Infection rates also were extremely high in both intersex phenotypes with 90% and 87% of male and female intersexes presenting infection, respectively.

Sexual phenotype	Co-infection	Paramyxean only infection	Microsporidian only infection	Uninfected	Total
Female Normal	10	8	1	30	49
Female Intersex	8	4	1	2	15
Male Normal	1	0	0	29	30
Male intersex	8	1	0	1	10

Table 8: Parasitic infection in *E. marinus* animals from Inverkeithing presenting a range of sexual phenotypes. Individual screen of infection rates in *D. duebenum* and Paramyxean parasites using the rDNA, 16S and 18S, respectively.

5.32 Transmission

PCR screening of pooled broods isolated from infected mothers revealed that Paramarteilia, *D. duebenum* and *D. berillonum* transmit their infection vertically to their offspring. The control broods in both individual and pooled screenings showed no infection. Seven broods from each infection group were screened. The parasite with the highest transmission efficiency was the paramyxean only infections, in which a mean of 96.8±2.1% of broods were infected from the mothers infection. The infection rate of the paramyxean slightly reduced in the co-infection group in which 93.7±2.5% of broods displayed infection, although, this was not significant (T-Value = 1.03 P-Value = 0.327 DF = 10).

Comparing the two microsporidian species, *D. duebenum* transmitted a higher percentage of infection (80.2±3.5%) than *D. berillonum* (64.5±2.6%) (see Table 9) which was statistically significant (T-Value = -3.347 P-Value = 0.012 DF = 7). The two females that did show *D. duebenum* only infections had 0% infection in their broods, indicating that the *D. duebenum* only accounts may not be a 'true' infection and possibly a contamination from the gut.

Brood sex ratio data from infected *D. berillonum* mothers gathered in chapter four showed a mean percentage of females in each brood being 41.5% and 55.0% for long (n=4) and short (n=2) day regimes, respectively. Comparing these values to the mean sex ratio of uninfected broods 38.47% (long day regime) and 56.89% (short day regime) showed no strong indication of feminisation. Infected broods showed an increase of females in long day regimes by 3.03% and an decrease in females in the the short day regimes of 1.89% which was equivalent to the range of sex ratios found in the uninfected groups within the experiment. Statisitical analysis failed to observe a significant difference between normal and infected broods (T-Value = 0.627; P-Value = 0.531; T = 662).

Brood infection % rates (n/total number of eggs or juveniles)			
Paramyxean only infection	*D. berillonum* infection	Co-infection	
		D. duebenum	Paramarteilia
100.0 (6/6)	73.9 (17/23)	66.7 (4/6)	83.3 (5/6)
100.0 (5/5)	66.7 (12/18)	91.3 (21/23)	100.0 (23/23)
100.0 (22/22)	64.3 (9/14)	72.7 (16/22)	100.0 (22/22)
94.1 (16/17)	68.2 (15/22)	76.7 (23/30)	96.7 (29/30)
100.0 (15/15)	57.1 (12/21)	83.3 (15/18)	94.4 (17/18)
94.7 (18/19)	66.7 (10/15)	92.0 (23/25)	96.0 (24/25)
89.0 (17/20)	54.6 (16/29)	78.6 (11/14)	85.7 (12/14)
96.8	**64.5**	**80.2**	**93.7**

Table 9: Brood infection rates of VT parasites found in the Langstone Harbour (*D. berillonum*) and Inverkeithing (Paramarteilia and *D. duebenun*) *E. marinus* population. Broods were tested via PCR methods to determine the transmission efficiency of parasite infection from mother to oocytes. Number of broods tested per infection group (n=7) and mean infection rate highlighted in bold.

5.33 Artificial infection

Individuals (ten males and ten females) from the Portsmouth population were infected by either feeding or injected of infection tissue dissected out of individuals from the Inverkeithing population. The control groups were infected with tissue from individuals in the Portsmouth population as sex distorting parasites are not present within this population. All experiment groups included were control fed, control injected, paramarteilia only injected, paramarteilia only fed, co-infection fed and co-infection injected. Horizontal transmission was only observed in the co-infection injected group (see Figure 38) following the four month incubation period, where the paramyxean was seen in 4 out of the 5 animals remaining (MI 1-5). In addition, one of the females (MI 2) was carrying late stage embryos, these were also screened and showed Paramarteila infection (MI 6). The paramyxean burden was higher in the females (MI1,2,4) than the males (MI3,5). *D. duebenum* showed no horizontal transmission in any of infection groups.

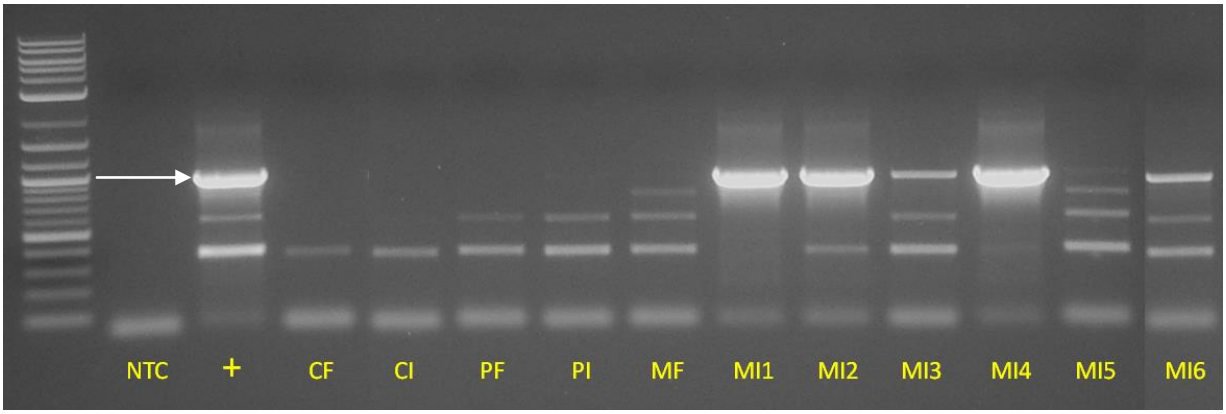

Figure 38: Screen for the presence of Paramartielia 18s rDNA gene in Langstone Harbour, Portsmouth sample population that were artificially infected either by feeding (F) or injecting (I) of various *E. marinus* tissue. Target product size is 905bp indicated by arrow. Control fed (CF) (n=7) and control injected (CI) (n=4) represents the pool of *E. marinus* that were artificially infected using tissue from other Portsmouth individuals (uninfected tissue). Paramarteilia only fed (PF) (n=7) and Paramarteilia only infected (PI) (n=4) represents the pool of *E. marinus* that were artificially infected with infected Paramyxean tissue from the Inverkeithing population. Co-infection fed (MF) (n=11) pool of mixed infection of *D. duebenum* and paramartielia tissue from the Inverkeithing population. MI 1-5 represents the individual screening of *E. marinus* that were artificially infected via injection of co-infected tissue. MI6 represents the brood that was harvested from the ovigerous female (MI2).

5.4 Discussion

To understand parasitic influences on crustacean sex determination and differentiation this study has highlighted two potential sex distorting parasites, the microsporidia *D. duebenum*, and an undescribed paramyxean that infects the *E. marinus* Inverkeithing population. Overall, 43% and 31% of the population were infected by the paramyxean and *D. duebenum*, respectively. Both parasites showed infection bias in the females and intersex phenotypes with 50% of females and 92% of intersexes being infected by at least one of the parasites. This indicates that one or both of the parasites are the cause of intersexuality and that some of the infected females were converted from genetic males which are reflected in the female bias infection prevalence. The brood infection prevalence (number of eggs that were in infected out of the total number of eggs in the brood) of infected females showed that females infected only by the paramyxid to have the highest transmission to the eggs (96.8%). This was reduced (93.7%) when the individual presented a co-infection with the microsporidia although was not statistically significant. *E. marinus* ovigerous females infected with *D. duebenum* transmitted the infection to 80.2% of the brood, a better transmission rate than the closely related *D. berillonum* which 64.5% of the host brood showed infection. Individuals that were infected with *D. duebenum* also showed a Paramyxean infection. The two accounts of *D. duebenum* only infections were possibly not 'true' infections due to its extremely weak signal in the PCR analysis, the lack of infection transferred to the broods and the prevalence of *D. duebenum* only infections within samples being rare. The weak infection of *D. duebenum* could possibly been due to contamination of the gut from ingested spores. Attempts to artificially infect *E. marinus* individuals from Langstone Harbour, Portsmouth population with the paramyxean parasite via injections, resulted in 4 out of the 5 surviving animals showing infection, with a greater infection burden observed in the females after 3 months post procedure. The infection also passed onto the broods of one of the females that were ovigerous. *D. duebenum* showed no signs of horizontal transmission in this experiment.

Microsporidia are a well documented group of parasites in which some species are thought to be sex distorters (Terry et al., 1999, Ironside et al. 2003). It has been

suggested that microsporidian infection causes intersexuality, as well as leading to complete sex reversal in male hosts. Within amphipods, the microsporidian *D. duebenum* has been classified as feminising parasites (Ironside et al. 2003, Terry et al., 2004). In this study we have found that an *E. marinus* population that has a high female bias and intersexuality (Ford et al., 2008, 2007) associated with *D. duebenum* infection has an additional species infecting this population that has been linked with sexual dysfunction in Crustacea, known as paramyxean. Paramyxean studies are mainly focused on the commercial economically important bivalves. The only non-mollusc reported to host paramyxean parasites are the amphipod, *O. gammarella* (Ginsburger-Vogel, 1991), the copepod, *Paracartia grani* (Carrasco et al., 2007) and the European edible crab, *Cancer pagurus* (Feist et al., 2009). This study to our knowledge is the second account of a paramyxean infecting an amphipod host. This is possibly due to a bias towards researching of more commercially viable species, rather than infection bias among Crustacean species.

The paramyxean parasite that is infecting the Inverkeithing population, in a manner similar to that seen for *D. duebenum* infection displays higher infection rates in the females and intersex phenotypes. This presents the question which is the feminiser or is it possibly both? The microsporidian and the Paramarteilia are vertically transmitting parasites and every individual found to be infected by *D. duebenum* also presented a co-infection with the Paramyxean. These two taxonomically divergent eukaryotic parasites with similar transmission strategy have extremely close association with each other suggesting a hitch hiking strategy might be at play. This co-infection between a Paramyxean and microsporidians have also been observed via light microscopy in the amphipod, *O. gammarella* (Ginsburger-Vogel, 1991). The stable co-occurrence of sex ratio distorters was thought to be rare phenomena due to theoretical predictions that the element with the highest basic reproductive rate should exclude other competitors (Bull, 1983). Several vertically transmitting microsporidian species, *Dictyocoela mulleri*, *Dictyocoela* sp., and *Nosema granulosis* infecting the same host population (*Gammarus roseli)* has shown extremely low co-infection in individuals (Haine et al., 2004). In addition, Hogg et al. (2002) found two vertically transmitting microsporidia species in a *G. duebenum* population which presented no co-infection within the study. However, multiple sex ratio distorters

with different evolutionary origins have been observed within their host (Ironside et al., 2003, Majerus et al., 2000).

In the artificial infection experiment, successful horizontal transfer was only observed following injection with co-infected tissue. Only the paramyxean horizontally transmitted into the injected *E. marinus* individuals, the parasite screen showed no evidence of infection from *D. duebenum*. This was also found when co-infected tissue was grafted in Orchestia which induced male intersexuality. However, the host tissue was examined and only Paramarteila cells could be found (Ginsburger-Vogel, 1991). This study found no signs of intersexuality or feminisation in the artificially infected individuals after the four month period. This could be due to insufficient time to develop characteristics or adult *E. marinus* cannot feminise and it is only through juvenile (earlier) stages that intersexuality can be developed. It should be noted there has been no accounts to our knowledge where *E. marinus* has sexually reversed or developed intersex features in laboratory conditions. However, both studies do suggest that the microsporidian might be a hitch hiking parasite within these amphipod species. In addition, paramyxeans can induce intersexuality in the amphipod host and therefore could have the capacity to feminise fully their host. The mechanisms that allow the paramyxean can horizontally transmit within *E. marinus* and *D. duebenum* cannot, remains uncertain. Previous studies of artificial infecting microsporidian Noesma sp. into an amphipod host has been successful with 3 out of the surviving 10 animals showed signs of infection (Dunn and Rigaud, 1998). Vertically transmitting Wolbachia have also shown the capacity of horizontal transmission (Rousset and de Stordeur, 1994). Whether vertically transmitting parasites horizontally transmit in natural populations is also open for debate. These studies indicate that sex distorting parasites do have the capability to transmit horizontally under laboratory conditions, although some species, such as *D. duebenum*, may have lost the capacity through their evolution.

Numerous attempts to infect organisms artificially with paramyxeans by co-inhabitation, feeding or injection have shown negative results (Balouet, 1979, Bethe et al., 1998, Van banning, 1979) with very few exceptions (Audemard et al., 2002,

Comps and Joly, 1980, Ginsburger-Vogel, 1991). Van Banning (1979) attempted feeding experiments of tissue infected with *M. refringens* to an *E. marinus* population in the Netherlands (described in the literature as *Marinogammarus marinus*) which showed no positive results, similar to this study. Horizontal transfer of Paramyxean appears to be not viable through ingestion; this could be simply that the spores pass through the gut without taking hold to host tissue or the period that the spores are in seawater before ingestion could cause spores to be ineffective. Audemard et al. (2002) successfully transmitted *M. refringens* from the oyster, *Ostrea edulis* to the copepod, *P. grani*, by simple co-inhabitation, this transmission was detected after 7 days of exposure to the infected oysters, although, the overall transmission was low and the attempts to reverse the transmission from copepod to oyster failed (Audemard et al., 2002, Carrasco et al., 2008). Another possibility is that the *E. marinus* individuals did not eat the tissue, although the starvation period of seven days alongside the cannibalistic tendencies of amphipods (Dick et al. 2005) makes this seem unlikely. *G. duebeni* was fed tissue infected with the microsporidian, *Pleistophora mulleri* which showed a 23% transmission efficiency when uninfected individuals were fed infected tissue (MacNeil et al., 2003). The same study also observed parasitised individuals were more likely to be cannibalised by both unparasitised and parasitised individuals. This further supports that transmission of parasites by feeding is possible in an amphipod host, although within this study we cannot be certain of why transmission this was not successful.

The paramyxean, *M. refringens* targets ovarian tissue in its copepod host *P. grani*, in which infected male copepods were never detected (Audemard et al., 2002). However, in mussels *M. refringes* infection rates and susceptibility showed no sex bias (Villalba et al., 1993). It would be interesting to determine whether *M. refingens* can sex distort its copepod hosts, or females are more susceptible to infection in horizontal transmission. Either scenario would support findings within this study. Interestingly, infection of ovaries by microsporidian parasites are also found in copepod hosts (Andreasis 1988, Micieli et al., 2000), although, co-infection of the two parasite groups have yet to be explored. Other paramyxeans to target gonads are found in *Orchestia gammarella* infected with *Paramarteilia orchestiae* and *Crassostrea gigas* infected with *Marteiliodes chungmuensis*. The only other

association between paramyxids and microsporidians that was identified in the literature was the microsporidia, *Noesma ormieresi* being described as a hyper-parasite of *M. refringens* (Comps et al., 1979 cited in Bethe et al., 2004). The microsporidian was observed to cause necrotic changes, such as, primary cell and sporangia degeneration, membrane alteration, cytoplasm condensation and reduction in number of spores. The microsporidian was suggested to be a possible biological control of marteiliosis sp., however, it has never been investigated further (Bethe et al. 2004). A histological or in situ florescence study into the spatial distribution of *D. dubenum* and the paramyxean in *E. marinus* adults and embryos would determine whether this is a simple co-infection occurrence or that the microsporidian acts as a hyper-parasite.

It is highly probable given that both the paramyxean and *D. duebenum* are vertically transmitting parasites that they have the same transmission strategy and therefore are not in direct conflict, in terms of host reproduction being of importance. Vertically transmitting parasites have to regulate their host exploitation due to their dependence on reproductive success. However, due to space and resource requirements from their host some form of compromise could be expected with the presence of both parasites. It has been observed *D. duebenum* can reduce other parasite species affects, such as, behavioural manipulation (Haine et al., 2005). In this study, a slight reduction in transmission efficiency (3.1%) was observed in the paramyxean species in the co-infected group compared with the paramyxean only infection group. In cases of co-infection in vertically transmitting *Wolbachia* strains it has been shown that infection intensities are reduced (Kondo et al., 2005). However, within this study there were no obvious differences in the strength of band between infection groups or parasite species. Although this finding was not statistically significant, it would be interesting to investigate the parasite burden within embryos of co-infected broods vs. paramyxean only broods via qPCR or immunoflourescent methods to see whether this slight reduction is due to reduced burden.

The findings in this study have indicated that *D. berillonum* is not a feminising agent which correlates with other studies (Terry et al. 2004). *D. berillonum* brood sex ratios

collect from the study in chapter 4 showed the mean percentage of females in each brood was 41.5% and 55.0% for long (n=4) and short (n=2) day regimes, respectively. Although, brood numbers are low, these findings do correlate with findings in chapter two that show no sex bias within the infection rates. This indicates this microsporidian species is not a feminiser and generally is a weaker vertical transmitter in *E. marinus* compared with the closely related, *D. duebenum*. Whereas, *D. duebenum* has been observed as a feminiser in past work (Ironside et al., 2003, Terry et al., 2004), a screen for paramyxea parasites was not conducted in these studies, therefore we cannot conclude this microsporidian species as a feminiser either. Interestingly, if *D. duebenum* is a sex distorting parasite, *D. berillonum* may have lost or *D. duebenum* have gained in their lineage the feminisation capability. However, due to its lack of transmission shown in the artificial infection experiment it could suggest that this species is well adapted in its vertical transmission; this is reflected in its high transmission to embryos, as well as, possible feminisation effects or hitchhiking strategy. This study has shown that the Paramyxean species infecting *E. marinus* has the capacity of horizontal and vertical transmission in laboratory conditions. We have not elucidated which parasite is the feminiser within the *E. marinus* population from Inverkeithing, we have identified two possible candidates, gained a better understanding of their transmission and demonstrated a successful artificial transmission model, so that further studies could be performed on a larger scale, in which an array of endpoints could be studied.

Paramyxean parasites have been within scientific literature for 40 years (Herrbach 1971). Despite this, there is a lack in knowledge of their taxonomy, pathology and life cycle, especially within less commercially important marine species. Although, some paramyxean species are well described in their basic morphology and cellular division, the literature still lacks comparisons among different species within this parasite group. The inconsistency within the taxonomy of the species has not assisted in this, for example, Paramyxea in the literature have often been incorrectly termed as Haplosporidians (Ironside et al. 2011). Further phylogenetic analysis based on DNA sequences will help the future taxonomy of this parasite group, more sequences need to be established in this phylum for greater comparisons among species.

Paramyxeans have been reported throughout the world (Bethe et al. 2004), yet very little information regarding the parasites ecological impact in the non-mollusc host has been established.

Elucidating the parasites responsible for the sex distortion observed in the *E. marinus* Inverkeithing population should be prioritised for future work. Co-infection in the population is high; however, the best strategy for finding the feminising agent would be to study individuals that are infected by one of the parasites. By artificially infecting females with paramyxean parasites, as *D. duebenum* appears not to transmit horizontally, this would leave Paramyxea only infected females. These females could breed and the brood sex ratios could be examined. If the infected females produced female biased broods this would indicate the paramyxean as a feminiser. However, this does not rule out *D. duebenum* having the capacity to feminise as well. Alternatively, the scenario could be that the paramyxean does not produce female biased broods. This would indicate *D. duebenum* as the feminising agent within the population. The female bias infection rates of the paramyxean observed in this study could be partially through the co-infection rates with the feminising microsporidia and the capacity of the paramyxean to transmit horizontally, in which, within the artificial infection experiment females showed greater parasite burden which possibly could indicate greater susceptibility. Either way, this is an interesting case of co-infection of two genetically and evolutionary divergent parasites in a female biased population, indicating that *E. marinus* is influenced by PSD. Further study, should include the identification of paramyxean parasites in other female biased populations, as well as, investigations into paramyxean-microsporidian interactions in other host populations. This will identify whether the Inverkeithing *E. marinus* population is an isolated case, provide further understanding of sex distorting parasites in an amphipod host, as well as, their role in sex determination and their influence over host population dynamics.

6. Identifying genetic sex determination in *Echinogammarus marinus*

6.1 Introduction

Sex differentiation is often caused by a sequence of gene expression events triggered by sex determination genes contained in sex chromosomes or autosomes (genotypic sex determination (GSD)). The majority of the time, genotypic or gametic sex determination fixes zygotic sex at the point of conception and the organism is set as that sex for the rest of its adult life (Charnov and Bull, 1977, Korpelainen, 1990). Sex determination in species that are heterogametic can be established by using Mendelian backcross experiments thus sex ratios are unbiased if mortality is not sex differentiated (Fisher, 1930). However, there can be factors that can overpower these genetic sex determining mechanisms. The general consensus when studying sex determination within crustaceans is that there are many sex differentiating mechanisms at play (Legrand and Legrand, 1987). However, a full understanding of these mechanisms and which factors override others still remains elusive. The aim of this study is to attempt to isolate genomic differences between the *E. marinus* genders in the hope of identifying the hetero or homo-gametic sex in *E. marinus*.

There are several methods able to detect genomic differences and sex determining mechanisms in Crustacea. Cytogentic investigations have shown that gammarid chromosome squashes are difficult to analyse due to small chromosome size, high chromosome numbers, and the presence of B chromosomes which are common among gammarids, including *E. marinus* (Orian, 1957) (see chapter one). Karylogical studies of gammarid species have seen little evidence for sex chromosomes; however this could be due to the technical difficulties in obtaining good quality metaphase plates, rather than the absence of sex chromosomes (Lecher et al. 1995). To overcome problematic karylogical investigations in crustaceans, female heterogamety can be demonstrated by crossing two genetic females, one of

which has been experimentally sex reversed to the extent of functioning as a neo-male. Genetic polychromatism is indicated when such a cross can produce all female viable broods. Experimental sex reversal has been accomplished by experimental removal or implantation of the androgenic gland (AG) in many crustacean species (Suzuki, 1999, Malecha et al., 1992) (see chapter 1) and has been achieved in the amphipod, *Orchestia gammarella* (Charniaux-Cotton, 1958, Charniaux-Cotton, 1960). Therefore, this study will not attempt to find sex chromosomes though karylogical methods, but attempt to ablate the AG from *E. marinus* male individuals and implant the AG in *E. marinus* females in hope of sexually reversing individuals for subsequent breeding experiments to determine the heterogametic sex.

This study will also attempt to use molecular methods to identify genetic differences between males and females. Sex specific molecular markers in crustaceans have mainly been identified in the commercially important decapods species (Ventura et al. 2011, Staelens et al. 2008, Perez et al. 2004, Zhang et al., 2007). Microsatellite also known as inter-simple sequence repeats (ISSRs), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLPs) analysis is typically used to study genetic variability within a population (Costa et al., 2004, Xu et al. 2001). However, these techniques have also been adopted to identify successfully genetic variation among genders in a variety of organisms (Gandolfi et al. 2001, Younis et al. 2008, Ventura et al. 2011). These three techniques attempt to identify differences between populations by separate approaches. RAPD is a type of PCR in which genomic DNA fragments are amplified completely at random and thereby able to differentiate genetically distinct individuals. Microsatellites are simple sequence repeats that occur in the coding and non-coding genomic regions. ISSR primers are designed to amplify the loci between two microsatellites. A common microsatellite is (CA)n repeat in which n differs between different alleles, this often reveals high levels of inter and intra specific variation and this can be observed by visualising different sized fragments on gel electrophoresis following a PCR reaction using the ISSR primers (Goldstein et al. 1995). The variability of microsatellites is due to the high mutation rate compared with other DNA regions. Therefore, if one gender has a unique genomic region, if may contain a distinctive microsatellite region that is detectable. The AFLP technique selectively amplifies

restriction enzyme digested genomic DNA. This usually consists of performing two enzyme digests using a rare and frequent cutter, followed by ligation of adapters for selective re-amplification which can then be visualised using high resolution gel electrophoresis (Vos et al., 1995). These three molecular marker techniques have been extended to many biological applications and can be extremely valuable as they do not require any prior knowledge of the genomic sequence of the target organism.

This study will screen pooled male and female *E. marinus* genomes using RAPD, ISSRs, and AFLP analysis to attempt to identify genomic differences between genders. In addition, the transcriptome of *E. marinus* has been sequenced, in which gonadal libraries of different sex phenotypes of *E. marinus* (normal male, intersex male, normal female intersex) were mapped using high through-put sequencing platforms (Roche 454 GS FLX and ABI SOLID). This has allowed the possibility of mining the transcriptome to identify genes presenting highly sex biased expression, which can be used as candidates for genes that might be genomically present in one sex.

The discovery of a specific genetic marker, and subsequent rapid molecular sexing assays would be extremely valuable for assessing whether sex genotype and phenotype corresponds in *E. marinus*. This would be particularly interesting in populations in which sex determination is highly influenced by parasitic, environmental, or pollution effects. It could also open up the possibility of being able to sex individuals before they are morphologically distinguishable, reducing time when conducting breeding experiments investigating sex ratios.

6.2 Materials and Methods

6.21 Androgenic gland sex reversal experiment

E. marinus were collected from beneath seaweed and rocks in the intertidal zone during low tide from Langstone Harbour, Portsmouth (see Figure 7). Animals were sexed and no intersex individuals were used. Animals were kept in aerated tanks with a 12 hour light regime at 10^{o}C. In gammarids the AG is located directly above the external genital papillae (see Figure 39). For the ablation of the AG, two techniques were adopted, burning of the genital papillae using a hot pin, in our attempt to eliminate the AG tissue, and dissecting out the bottom area of the testis to remove the area in which the AG is located. 36 individuals were used per method with an addition control ablation group (control burning and control dissection) in which the burning or removal of tissue was conducted in an area slightly above the genital papillae, so not to affect any of the reproductive tissue. An implantation experiment was also conducted in which female *E. marinus* had a small incision and had testis tissue (containing the AG) implanted into the area where the AG normally resides in males. The control group had muscle tissue implanted into females in the same area. After ablation or implantation was conducted, animals were put in aerated water and kept in separate plastic pots to avoid cannibalism. After 3 moults animals were anaesthetised and checked for signs of feminisation (brood plates) or masculinisation (genital papillae). If signs of sex reversal were observed, individuals were left till subsequent breeding with the same sex could be achieved. Broods would be kept and sex ratios would be identified once juveniles could be sexually differentiated. These sex ratios can then indicate which gender is the heterogametic sex.

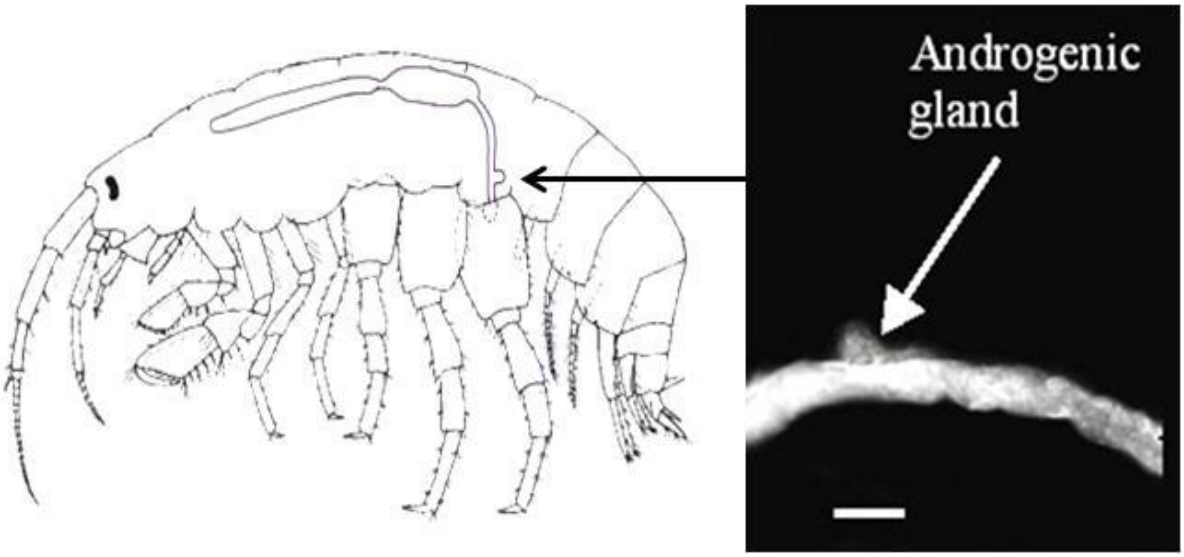

Figure 39: The androgenic gland (arrows) in *Echinogammarus marinus*.

6.22 Transcriptome mining for potential sex markers

Recently the transcriptome of *E. marinus* has been sequenced (unpublished), in which gonadal libraries of different sex phenotypes of *E. marinus* (normal male, intersex male, normal female, intersex female) were mapped using high through-put sequencing platforms (Roche 454 GS FLX and ABI SOLID). A total of 19 and 15 genes presenting extreme female and male exclusive expression respectively were selected and primers designed using primer 3 (Primer BLAST, NCBI) (see Table 10). Ovary and testis were dissected from normal male and female *E. marinus* from the Langstone Harbour, Portsmouth population. The cDNA libraries were made in accordance with methodologies in Section 3.23. Initially, the primers were tested by performing PCR using the cDNA libraries as template and analysing the products by agrose gel electrophoresis. This validated the expression suggested by 454 and SOLID sequencing and established suitable PCR conditions. Genes that showed potential for being sex specific were then tested using pooled genomic DNA extracted from 250 males and 250 females pools obtained from the Langstone harbour, Portsmouth population (DNA was originally obtained for the parasite screen described in chapter 2) to examine if the gene was sex specific or present in both genders. All PCR reactions were in 25µl volumes containing 2.5 mM $MgCl^2$, 0.25 mM each deoxynucleotide, 0.5 mM each primer, 1 x PCR buffer 1U Taq DNA polymerase, 1µl (10ng) of template DNA (Promega). PCR conditions consisted of an initial denaturation at 94°C (4 minutes), 35 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (45 seconds), a final incubation of 5 minutes at 72°C. The product was then resolved using a 1.2% agrose gel containing ethidium bromide (4%) that was run for 20 mins at 150V before being visualised under a UV transilluminator.

Female Biased Expressed Genes		Product Size (bp)	Male Biased Expressed Genes		Product Size (bp)
Primer	**Primer Sequence ('5-'3)**		**Primer**	**Primer Sequence ('5-'3)**	
F3	TCGTGGCGTTCACCGACCATG GACCATGGGACCGCCATTGGC	188	M4	TGCTGAGGGCACCCTTTGTGT GTTGTGCGAGCGTCCTCCCC	161
F4	GCAGCGGCCACACACCATAGT AGCTGAGAAACTGCACGCGTC	165	M5	CGCGCTCTAGGGTTGAAGCTC GCAAACCTGCTTCTGCTTTATATC	102
F5	GGAGCTCGTGTAGTGAGCCACG TGAGCAGCCCGCATGCGTAG	192	M6	GAGGGACATCAATGTTCAAGTGTC CGTTGATCAAGCACCTGGTAGCG	102
F6	CGCAGCGGCGACTTGCTTATC GCGTCTTGCATGCTGTCATGACC	200	M7	GCACACAAGTCAGGGCCGCT AGCGGCCCTGACTTGTGTGC	190
F7	AAAGAGCCGACAACCGGCAGC TCGTGACCGCTTTTAACGGCC	155	M8	TGCGTGGGTGCAGTCCTCAC CACCGCGGCGACCTGTGTTA	160
F8	CGTCGGGCAGGCAAGGTCTG GGATTTTGGAGGATGGACCGCCG	150	M9	CCGATGCCCTTCGCATCCCAG ACCAGCCACTTTCCCCCACGT	150
F9	CATCGGAAGGCATGTCACGCGT CGCCCTCGCTGCTGTTTGCTA	155	M10	GCAGTTACGGCGCAGGAAGTT TGTGGCATGTGGCACCGTCTTG	175
F10	GCTCTCGACGACACCCCAGTT ACGCGGATGCGCAAGATGGTAA	191	M11	GCCAGTCGGTAAGTGGCGGC CTTGCGGCGACTCCCTCTGC	195
F11	GGCCCAACCGAGGATGAGCC GCGGTCGGTAGTACGGTTGGT	169	M12	CTCGGCCATTGCAGCCCCA GATCGCGCCGACACCGGTAT	196
F12	CGACTACCTCTTACCAGGGGCACC GCACGGAAACCCCCGTCGAG	153	M13	TCCGGCGAGGTTTTGGGTCG TCTTCCGGCCAGGCTTTGGG	196
F13	TCAGAGTGGGTCATCGGGCGA AGGCCCCGAGATGCCGCATA	157	M14	GGCCGCCGTATTACTTCTCA GTCGGTAACACCTGAAGCCA	171
F14	ACTGGCATCGCCACCCAAAC TTGAGCACACCACGTCGCGTT	167	M15	AGCGAAACGCGTCATGGATA CACTTATTCGCGGCCACTTG	229
F15	TACCCTGGTCCCCATCGGCC GGAGCGCTGCACGCCGATAT	160	M16	GTTCCTAGTTGGGTGGACGG TGCGACTATTCGGCCTGTTT	134
F16	AGCAAACACTCTGGAGTCCG CGAGTACGGCTGTCGCTATT	170	M17	GATCAGTTTGCAGCAAGGGC ACCCCTGCAATAGAACGACG	152
F17	AGCCGTTCGTGACCTACAAG TTGGCATCCTCTACCGCAAG	196	M18	AGCTGAATCACGAGCGAGTT TGCCTACCTTCGGTCTACCA	121
F18	TCGCTGGTAACCCACTTCAC TGGAAGAGTTTTCCCTCGGC	148	M19	CTCGACGAATGAGGTCTCGG GTGGAAGTCTGTACTCGGGC	151
F19	GGTATGGAGCTGACGATGGG TTCCCTTTCCAATCGCGTCA	133			
F20	TTCGACCCGTCTACTTTGGC GTTCCGCAGTTGGTACTGGA	153			
F21	CCCAGCCTTGTTGAGGAAGT ACGTAACCGAGTCCCCTGTA	227			

Table 10: Primers for contiguous sequences presenting sex exclusive expression: primer sequence and product size.

6.23 RAPD and ISSR PCR Techniques

Male and female pooled gDNA, originally extracted for the parasite screening in the population study (chapter 2) was used (250 individuals per gender) from the Langstone Harbour population. 20 RAPD (OPE1-20, Eurofin MWG Operon's RAPD 10mer E Kit) (see Table 11) and 25 ISSRs (Eurogentec, UK) (See Table 12) were tested and amplification was performed in 25 µl volume containing, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.1 mM primer, 1 x PCR buffer, 1U Taq DNA polymerase (Promega) and 25 ng pooled genomic DNA. PCR conditions consisted of an initial denaturation at 94°C (5 minutes), 35 cycles of 94°C (30 seconds), 32°C (40 seconds), and 72°C (45 seconds), a final incubation of 10 minutes at 72°C. The ISSR primers had adjusted annealing temperature (38-64^{o}C) to improve resolution (See Table 12). The product was then resolved using a 1.2% agrose gel containing ethidium bromide (4%) that was run for 40 mins at 150V before being visualised under a UV transilluminator.

RAPD	
Primer name	Primer sequence (5'-3'
OPE-01	CCCAAGGTCC
OPE-02	GGTGCGGGAA
OPE-03	CCAGATGCAC
OPE-04	GTGACATGCC
OPE-05	TCAGGGAGGT
OPE-06	AAGACCCCTC
OPE-07	AGATGCAGCC
OPE-08	TCACCACGGT
OPE-09	CTTCACCCGA
OPE-10	CACCAGGTGA
OPE-11	GAGTCTCAGG
OPE-12	TTATCGCCCC
OPE-13	CCCGATTCGG
OPE-14	TGCGGCTGAG
OPE-15	ACGCACAACC
OPE-16	GGTGACTGTG
OPE-17	CTACTGCCGT
OPE-18	GGACTGCAGA
OPE-19	ACGGCGTATG
OPE-20	AACGGTGACC

Table 11: RAPD primers used in the attempt to identify genetic differences between male and female *Echinogammarus marinus*.

ISSR		Annealing temperature (^{o}C)
Primer name	**Primer sequence ('5-'3)**	
7	CTCTCTCTCTCTCTCTRG	58
814	CTCTCTCTCTCTCTCTTG	54
843	CTCTCTCTCTCTCTCTRA	56
844	CTCTCTCTCTCTCTCTRC	58
898	CACACACACACARY	42
899	CACACACACACARG	46
901	GTGTGTGTGTGTYR	42
902	GTGTGTGTGTGTAY	44
AW3	GTGTGTGTGTGTRG	46
Becky	CACACACACACACAYC	52
Chris	CACACACACACACAYG	52
DAT	CACACACACACACARG	52
Goofy	GTGTGTGTGTGTGTYG	52
John	AGAGAGAGAGAGAGYC	52
M1	CAAGAGAGAGAGA	38
M2	GGGCGAGAGAGAGAGAGAGA	64
Mao	CTCCTCCTCCTCRC	50
Manny	CACCACCACCACRC	50
OMAR	GAGGAGGAGGAGRC	50
Sas 1	GTGGTGGTGGTGC	44
Sas 2	GAGGAGGAGGAGC	44
Terry	GTGGTGGTGGGTGRC	50
UBC809	AGAGAGAGAGAGAGAGG	52
UBC811	GAGAGAGAGAGAGAGAC	52
UBC827	ACACACACACACACAC8G	52

Table 12: ISSR primers and their associated annealing temperatures used for microsatellite analysis between male and female *Echinogammarus marinus*.

6.24 Amplified Fragment Length Polymorphism (AFLP)

An AFLP comparison was performed using male and female pooled DNA from the Langstone Harbour population (250 individuals per gender), originally extracted for the population parasite screening described chapter 2. AFLP markers were obtained using a one-step digestion–ligation procedure based on methodology detailed in Vos et al. (1995) with a few modifications. DNA (250 ng) was digested and ligated in the same reaction for 3 hours at 37^{o}C in solution containing 4 pmol of EcoRI adaptor (Eco-F: 5'-CTC GTA GAC TGC GTA CC-3', Eco-R: 5'-AAT TGG TAC GCA GTC TAC-3'), 50 pmol of MseI adaptor (Mse-F: 5'-GAC GAT GAG TCC TGA G-3', Mse-R: 5'-TAC TCA GGA CTC AT-3') 10 mM of ATP, 1 U of T4 ligase (NEB), 5U of MseI (NEB), 5 U of EcoRI (NEB) and water to complete 10 µl per sample. After digestion–ligation, samples were diluted 1:10 with molecular grade distilled water. Pre- and selective PCR amplification was carried out with PCR reagents (Promega). The PCR reactions contained 1x PCR buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of dNTPs and 0.008 U/µl of Taq polymerase. Pre-amplification was carried out with 5 µl of the ligated product and 0.2 µM of both EcoRI (5'-GACTGCGTACCAATTCA-3') and MseI (5'-GATGAGTCCTGAGTAAC-3’) preselective primers in a total volume of 25 µl. Selective amplification consisted of performing PCR with the pre-selective primers consisting of 3 or 4 randomly added nucleotides (See Table 13). In total, 36 primer combinations were used to analyse the gDNA. PCR conditions consisted of an initial denaturation at 94°C (2 minutes), 35 cycles of 94°C (30 seconds), 54°C (60 seconds), and 72°C (60 seconds), a final incubation of 2 minutes at 72°C.

EcoR1 selective Primer	Added Nucleotides	MSEI Selective Primer	Added Nucleotides
Eco 1	AGCC	MSE 1	CCAA
Eco 2	AGAC	MSE 2	CCAC
Eco 3	AATT	MSE 3	CCCC
Eco 4	GGC	MSE 4	TCC
Eco 5	CCA	MSE 5	TGG
Eco 6	GAC	MSE 6	CCG

Table 13: Selective amplification primers for AFLP analysis with 3 or 4 (xxx/x) nucleotides added to ensure reduced band profiles in analysis (ECOR1- 5'-GACTGCGTACCAATTCA-XXX/X-3')(MSEI- 5'-GATGAGTCCTGAGTAAC-XXX/X-3').

Initially, the AFLP markers comparing male and female gDNA libraries (n=250) were tested and the product was then resolved using a 1.2% agrose gel containing ethidium bromide (4%) that was run for 40 mins at 150V before being visualised under a UV transilluminator (see Figure 40). Then, 15 primer combinations presenting various DNA fragments showing variation between genders were selected for further analysis using radioactive labelling and polyacrylamide gel electrophoresis for better resolution of the potentially sex specific DNA fragments.

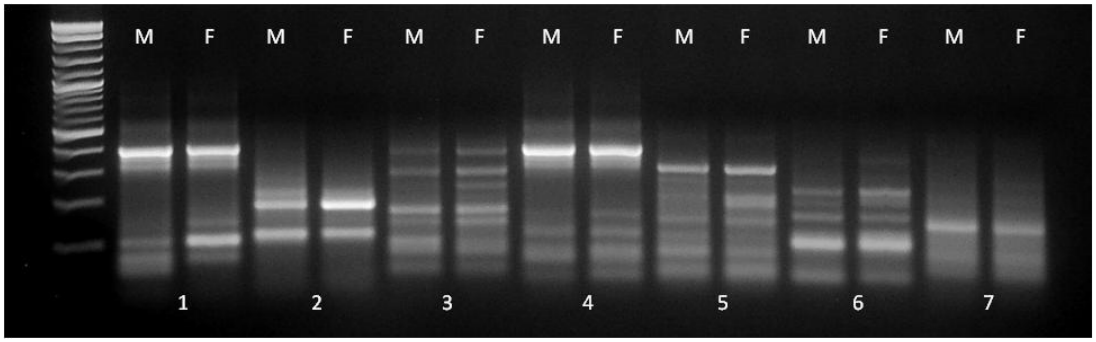

Figure 40: Representational sample of PCR analysis of selective primer combinations using male (M) and female (F) *Echinogammarus marinus* gDNA pools (n=250) to select primer combinations (1= eco2, mse5, 2= eco3, mse6, 3= eco2, mse3, 4= eco1, mse2, 5= eco3, mse4, 6= eco1, mse3, 7= eco1, mse5) that produces a variety of different sized DNA fragments for further analysis using polyacrylamide denaturing gels and radioactive labelling.

The conditions for radiolabelled PCR were the same as described above except the PCR master mix was spiked with 1µl of α 32P dATP. The subsequent PCR products (12 µl per sample) were separated by vertical electrophoresis (Sequi-Gen GT electrophoresis system, Bio-Rad) in 1-mm thick denaturing gel containing 6% acrylamide/bisacrylamide mixture (16:1) (Sequagel 6, National Diagnostics) and 600 µl 10% APS. The gel was pre-run at 65 W for 1 hour to heat gel up to running temperature before sample was loaded. The gel was then run for a further hour with the samples loaded. Gels were fixed in an 10% acetic acid bath for 10 mins, transferred onto Whatmann 3MM paper and covered with 'Saran' wrap, prior to being dried at 80^{o}C for 1 hour under a vacuum. The radioactive labelled fragments were then visualised using a Fujifilm FLA 5000 phosporimager. Bands identified as being sex specific were cut out using a razor blade. The excised gel fragments were then heated up to 37^{o}C, homogenised in TE buffer and left on a shaker over night. The solution was then centrifuged at high speed (14,000 rpm) for 5 minutes and supernatant was kept and used for re-amplification using the original PCR conditions. The re-amplified DNA was visualised using agrose gel containing Gel Green (Cambridge Bioscience, UK). The bands were then cut and eluted using QIAquick gel extraction kit (Qiagen, UK) and sequenced using the Sanger method (Source Bioscience, UK). Sequences were analysed using BLAST (NCBI) for possible annotation and primers were designed based on the newly generated sequences using Primer 3 (Primer BLAST, NCBI). Primers were then tested on male and female gDNA to validate their use in detecting a genomic marker for sex. All PCR reactions were in a 25µl volumes containing, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 mM primer, 1 x PCR buffer, 1U Taq DNA polymerase (Promega) and 1µl (10ng) of template DNA. PCR conditions consisted of an initial denaturation at 94°C (4 minutes), 35 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (45 seconds), a final incubation of 5 minutes at 72°C. The product was then resolved using a 1.2% agrose gel containing ethidium bromide (4%) that was run for 20 mins at 150V before being visualised under a UV transilluminator.

6.3 Results

6.31 Androgenic gland sex reversal experiment

All groups had high mortalities rates with the exception of the control group that showed 19% mortality over the three month period (see Table 14). The group that had their AG ablated via burning the area with a hot pin had the highest mortality. There was no difference in mortality between the control and test groups. Overall after three moults there was no sign of feminisation in the AG ablated groups or masculinisation in the AG implanted groups. Therefore no further breeding experiments were conducted to elucidate which gender was the heterogametic sex in *E. marinus*.

Group	Mortality (x/36)	Mortality (%)
Ablation (dissected)	29	81
Control (dissected)	28	78
Ablation (burnt)	33	92
Control (burnt)	34	94
Implanted	27	75
Implanted control	28	78
Control	7	19

Table 14: Mortality rates of *Echinogammarus marinus* individuals in the androgenic gland sex reversal experiment

6.32 Transcriptome mining for potential sex markers

The transcriptomic data generated using the both 454 and SOLID platforms identified 15 male and 19 female sex specific gene candidates on the basis of their sex exclusive expression. The high through-put sequencing performed using the Roche 454 (GSFLX) platform identified 13 female and 10 male gene candidates (see Table 15). Later on, more transcriptomic data was sequenced based on the SOLID ABI platform with greater reading depth (see Table 16), this allowed for the gene candidates chosen to be compared with the new sequencing data and a further 6 female and 5 male exclusively expressed genes were designed using primer BLAST (NCBI) (see Table 10) and tested. Genes were selected with 0 number of reads indicating the potential for no expression in either normal males or females in hope to find a genomic marker present in a single gender.

From the transcriptomic data mapped 29538 genes showed bias expression in females and 2149 genes showed bias expression in males, of which 698 and 259 of those genes were annotated, respectively. Out of 34 gene candidates selected 11 were annotated, 9 of which were female biased and 2 were male biased in their expression (see Table 17). From ovary and testis cDNA libraries created PCR analysis showed good validation of the transcriptome data (see Figure 41). Although, when testing the primers using gDNA all PCR analysis showed presence of the gene in both genders (see Figure 42).

Primer	Female Biased Genes				Primer	Male Biased Genes			
	nf	if	nm	im		nf	if	nm	im
	Number of reads					Number of reads			
F3	44	41	0	0	M4	0	0	586	223
F4	36	37	0	0	M5	0	0	261	130
F5	26	30	0	0	M6	0	0	256	133
F6	21	12	0	0	M7	0	0	185	175
F7	16	20	0	0	M8	0	0	178	33
F8	15	20	0	0	M9	0	0	29	23
F9	36	37	0	0	M10	0	0	23	17
F10	53	62	0	0	M11	0	0	19	17
F11	52	196	0	0	M12	0	0	26	14
F12	38	39	0	0	M13	0	0	169	112
F13	9	8	0	0					
F14	6	11	0	0					
F15	11	11	0	0					

Table 15: Expression (reads in expressed sequence tags (ESTs)) of genes in gonads isolated from normal females (nf), intersex females (if), normal males (nm) and intersex males (im) generated by high throughput sequencing using the ROCHE 454 GSFLX platform. Sequences presenting extreme sex biased expression selected as candidate genomic markers.

Primer	Solid RNAseq					
	IFIN	NFIN	NFUN	IIMUN	NMUN	EIMIN
	No. reads					
F16	4890	2651	3299	0	0	3
F17	1050	1181	1733	0	0	2
F18	1150	562	1712	2	0	3
F19	3045	2127	1699	1	0	0
F20	2182	1884	1607	2	0	1
F21	848	571	1438	0	0	0
M14	4	1	0	708	571	554
M15	0	1	0	117	480	339
M16	2	2	0	520	461	593
M17	2	2	0	859	408	552
M18	2	2	0	382	387	344

Table 16: Expression (reads in expressed sequence tags (ESTs)) of genes in gonads isolated from *E. marinus* categorised by sexual phenotypes and microsporidian infection. Normal females uninfected (NFUN), normal females infected (NFIN), intersex females infected (IFIN), normal males uninfected (NMUN), internal intersex males uninfected (IIMUN), external intersex males infected (EIMin). The data was generated by high throughput sequencing using the SOLID ABI platform. Sequences presenting extreme sex biased expression selected as candidate genomic markers.

Primer	Swissprot ID	Description	eValue
F3	HSP7C_CHICK	Heat shock cognate 71 kDa protein	1E-136
F6	GOR_PANTR	Exonuclease GOR	2E-15
F7	GOR_HUMAN	Exonuclease GOR	2E-20
F8	PLMN_CANFA	Plasminogen (Fragment)	5E-11
F9	NDUAB_MOUSE	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	5E-12
F10	PNO1_NEMVE	RNA-binding protein pno1	9E-63
F13	NANOS_DROME	Protein nanos	0.000000002
F15	CISD2_DROPS	CDGSH iron-sulfur domain-containing protein 2 homolog	4E-18
F17	PGCP_MOUSE	Plasma glutamate carboxypeptidase	6E-25
M11	ATD1A_DANRE	ATPase family AAA domain-containing protein 1-A	3E-59
M12	POL2_DROME	Retrovirus-related Pol polyprotein from transposon 297	4E-78

Table 17: Annotated genes selected for possible sex specific biomarkers.

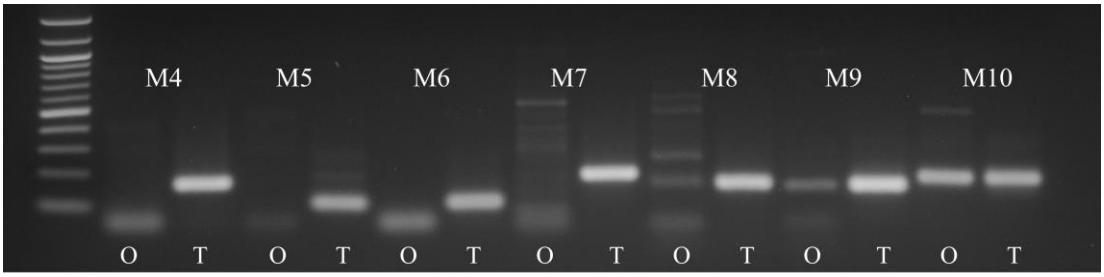

Figure 41: Representation sample of PCR analysis and validation of primers (M4-10) to amplify genes presenting male bias expression using cDNA libraries of *Echinogammarus marinus* ovaries (O) and testis (T) alongside a 2 log ladder (NEB).

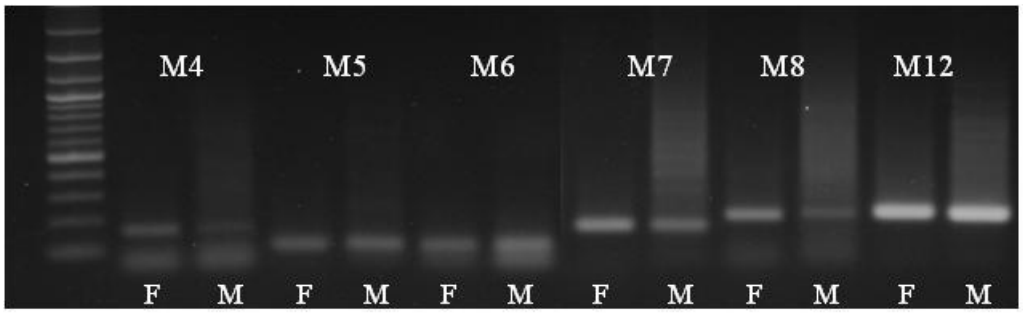

Figure 42: Representation sample of PCR analysis of primers (M4-8, M12) to amplify genes presenting male bias expression using gDNA pools of *Echinogammarus marinus* females (F) and males (M) alongside a 2 log ladder (NEB).

6.33 RAPD and ISSR PCR techniques

PCR analysis showed that no sex specific genomic regions were observed in either the RAPD (see Figure 43) or ISSR primers (see Figure 44) that were investigated. Therefore DNA fragments that were amplified by the primers showed no differences in the banding patterns between the gender pools tested. Analysis was originally conducted using pooled gDNA of 250 animals per gender library (see Figure 43 and 43) and gDNA taken from individuals (see Figure 45). The individual analysis did show that animals from the Langstone Harbour population exhibited genetic variation although, this wasn't associated with sex.

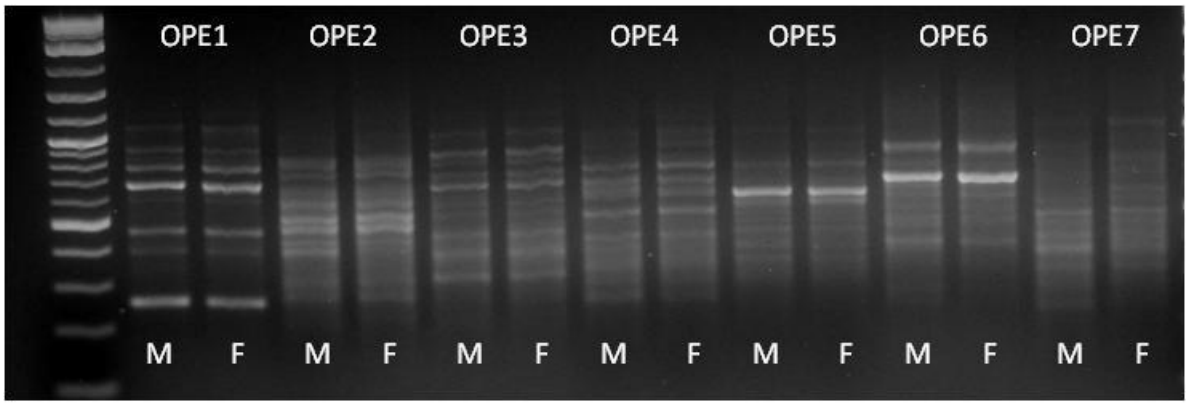

Figure 43: PCR analysis using RAPD primers (OPE1-7) with pooled male and female *Echinogammarus marinus* libraries alongside a 2 log ladder (NEB).

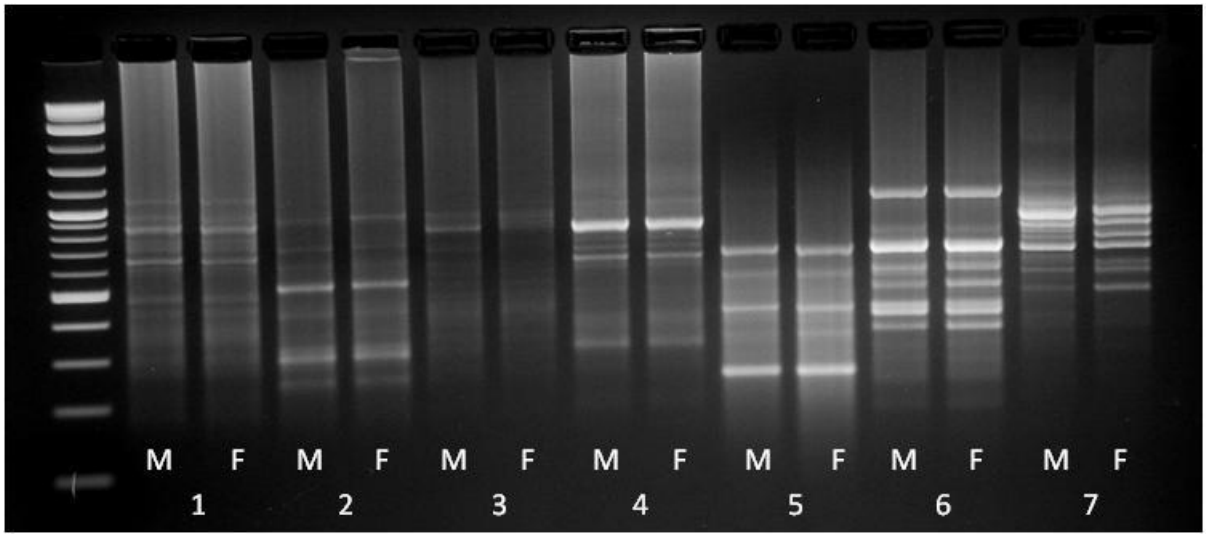

Figure 44: PCR microsatellite analysis using ISSR primers (1= Becky, 2 = 901, 3 = 902, 4 = UBC 801, 5 = Terry, 6 = 814, 7 = 843) with pooled male and female *Echinogammarus marinus* libraries alongside a 2 log ladder (NEB).

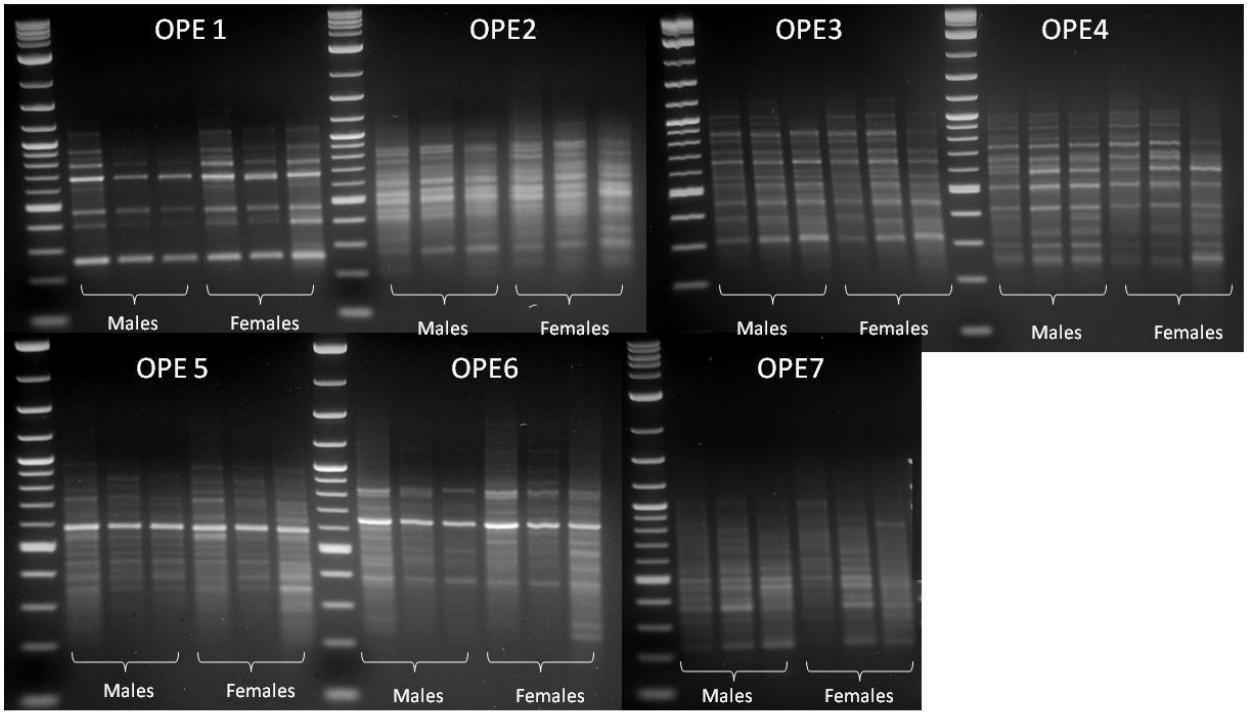

Figure 45: Representative sample of PCR analysis using RAPD primers (OPE1-7) with gDNA taken from *Echinogammarus marinus* individuals (3 males and 3 females) alongside a 2 log ladder (NEB).

6.34 Amplified fragmented length polymorphism (AFLP)

Following AFLP analysis, 15 primer combinations were selected for investigation. Sex specific bands were detected (see Figure 46) and sex specific genomic regions were cut out and sequenced using the Sanger method. The majority of the bands that were sequenced showed mixed signal within the reads, meaning that duplicate DNA fragments were within the sample and therefore a sequence could not be retrieved. In addition, several DNA fragments that were isolated from the lower regions of the gel could not be sequenced due to their small size. In total, 4 bands produced relatively clean sequences, two were male specific and two were female specific. BLAST search analysis against sequences stored in GenBank (NCBI) indicated no annotation was possible for the generated sequences. Primers were designed to verify whether the DNA fragments were sex specific by performing PCR analysis using individual *E. marinus* gDNA samples. However, none of the sequences tested showed sex specificity following the PCR validation (see Figure 47).

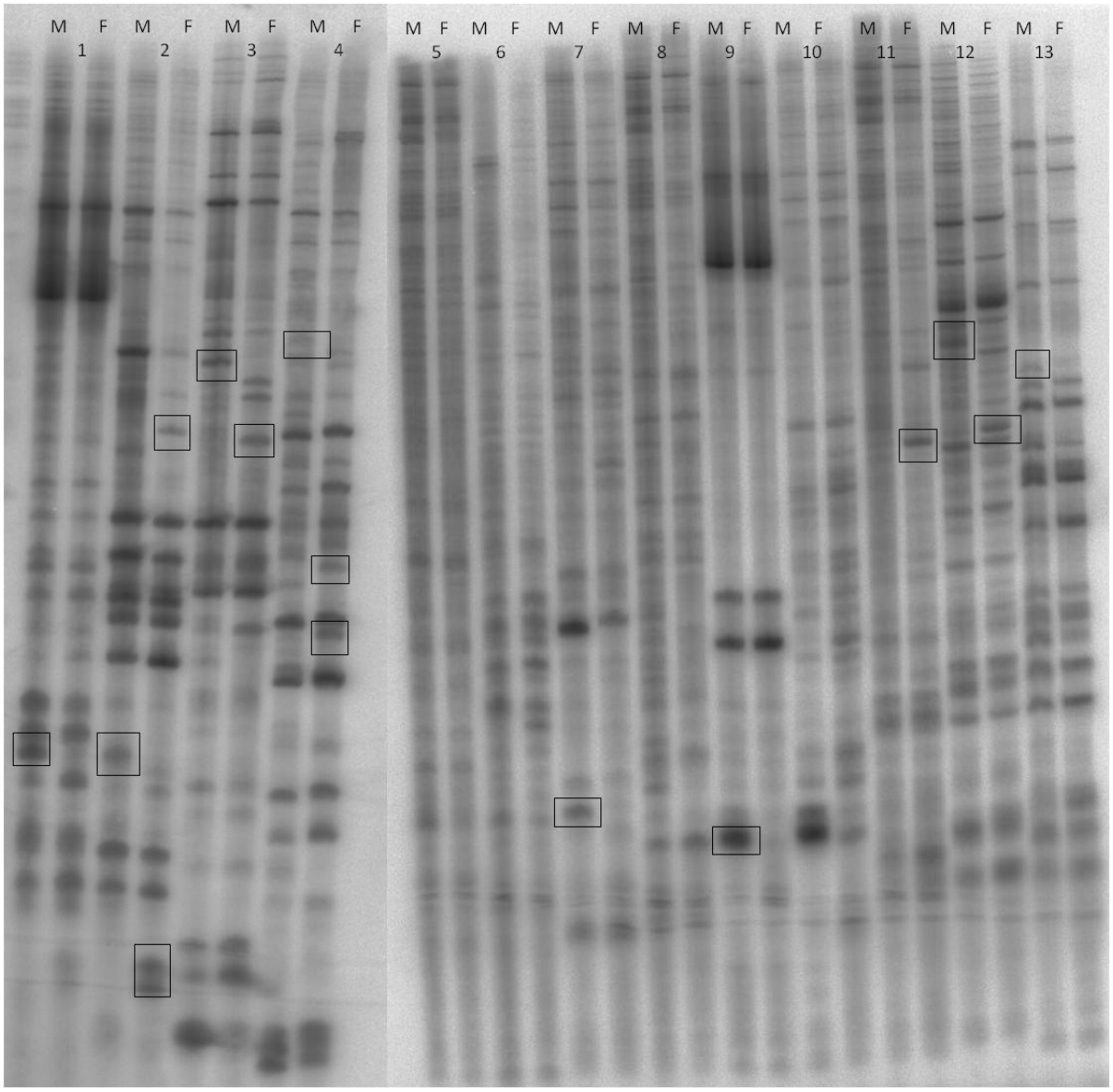

Figure 46: Polysacrylamide vertical gel electrophoresis with 32P labelled PCR product using male (M) and female (F) *Echinogammarus marinus* gDNA pools (n=250) following AFLP analysis. Sex specific bands highlighted by black boxes, primers used for PCR reactions 1= eco1, mse6, 2= eco1, mse3, 3= eco1, mse2, 4= eco2, mse3, 5= eco6, mse2, 6= eco5, mse4, 7= eco5, mse3, 8= eco4, mse2, 9= eco4, mse6, 10= eco4, mse3, 11= eco4, mse2, 12= eco1, mse2, 13= eco3, mse1.

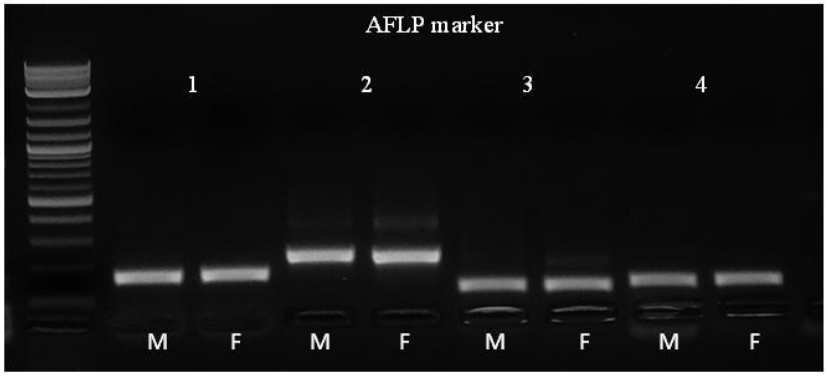

Figure 47: Validation of potential sex specific markers generated from the AFLP analysis (1-4), female specific (1 and 2) and male specific (3 and 4) showed no variation between *Echinogammarus marinus* genders (m = male, f = female) alongside a 2 log ladder (NEB).

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6.4 Discussion

This study employed a variety of techniques in an attempt to isolate sex specific regions of DNA as well as trying to establish which gender was the heterogametic sex in *E. marinus*. However, none of the techniques employed achieved these aims. The absence of evidence in these investigations does not necessarily indicate that the evidence is absent; therefore we cannot currently make conclusive statements regarding GSD in *E. marinus*. However, extensive karylogical searches have found little evidence of sex chromosomes within amphipods (Libertini and Rampin, 2009, Orian, 1957) and in conjunction with the failure of this study, raise the question of whether *E. marinus* sex determination mechanism could be solely environmental or parasitic. Sexual phenotypes within *E. marinus* show great plasticity and intersexuality is common in some populations (Ford and Fernandes, 2005), with studies indicating it might be caused by a variety of parameters such as ESD, pollution and parasitic influences (Ford et al. 2006, Guler et al. 2012, Short et al., 2012b).

The AG manipulation experiments did not successfully result in sex reversal or any sign of intersexuality in the surviving individuals. In addition, mortality was high and ranged from 74-94 % over the three month experiment period. The group that had their AG ablated through burning the area with a hot pin had the highest mortality rates. This was likely due to difficulties in not damaging the surrounding area by this method as the hot pin occasionally would come in contact with non target tissue. Dissecting the tissue area was less harmful to the animal but still presented high mortality. The failure of this experiment could have been due to the insufficient implantation or ablation of the AG possibly due to the small size of individuals, their ability to recover, or the assumed location of the AG within *E. marinus* being incorrect. Alternatively, *E. marinus* may not have the capacity to induce intersexuality or sex reversal as an adult. Indeed, experimental sex reversal has not been achieved in this species to our knowledge. High levels of intersexuality and skewed sex ratios in natural populations of *E. marinus* do suggest that the species has great sexual plasticity (Ford et al., 2004). However, the flexibility in their sexual

differentiation may only be possible during early developmental stages and as an adult their sex is largely or completely fixed. The only successful AG manipulation experiments that have induced full sex reversal in adult amphipods have been conducted on *Orchestia gammerella* in the 1950's (Chaniaux-cotton, 1958, 1960). However, this approach has also been successfully applied in many decapod and isopod species to show heterogamety (Malecha et al. 1992, Suzuki, 1999).

Several PCR based techniques were attempted in this study to identify a genetic sex marker in *E. marinus*. RAPD and ISSR-PCR analysis showed no evidence of genetic variation between the sexes with a total of 45 primers tested on male and female pools. Costa et al. (2004) conducted RAPD analysis on three gammarid species, *Gammarus locusta, Gammarus chevreuxi and Gammarus insensibilis,* and found no sex specific markers within the study, although only ten RAPD primers were tested. One RAPD primer can only amplify several thousand base pairs of genomic sequence therefore, to identify small genomic differences between genders may require more RAPD primers than used within this study. Successful studies that have isolated sex specific markers have used a greater number of RAPD primers. For example, in the common carp, one male specific DNA fragment was identified after testing 220 RAPD primers using pooled DNA (Chen et al. 2009). Obtaining a target marker also relies on chance, as the sequences amplified by these random primers are not known. In addition, the size of the genomic region of interest, as well as the overall genome size, are all factors influencing the likelihood of success. Further testing would be required to comment on the usefulness of RAPD and ISSR analysis for determining genetic sex differences in *E. marinus*. However, non-sex specific individual variation was observed showing that these ISSR and RAPD analysis would be a useful tool in population genetic studies.

The AFLP analysis showed much more promise, with obvious genetic differences evident by the band patterns in the highly resolved polyacrylamide gels. Several studies have successfully used AFLP techniques to identify sex specific genomic differences in crustaceans (Ventura et al. 2011, Staelens et al. 2007, Zhang et al., 2007). In this study, the majority of bands sequenced had mixed signal, indicating

more than one sequence was in the banding area. However, the large number of potential sex markers identified by this technique indicates that further development of methodology is required. One possible solution to prevent mixed signal when sequencing sex specific fragments would be to clone the DNA fragments generated. Alternatively, increasing the selective nucleotides to 5 or 6 on the amplifying primer would result in less DNA bands/fragments being generated, reducing the likelihood of overlapping DNA fragments being sequencing. Several sex specific fragments were too small for sequencing, genome walking to reveal sequences that fall outside the AFLP bands generated could be used (Brugmans et al. 2003). Although, this investigation did not identify a genetic sex marker, it did highlight possible genetic variations between sexes.

Transcriptomic mining for genes that showed sex exclusive expression revealed a greater number of candidates in females than males. Although this is purely speculative, this may reflect a greater variation in types of cells found in the ovaries compared to the testis. The initial investigations using gonadal cDNA revealed the genes that showed male exclusive expression displayed greater potential, with more male marker candidates exhibiting no expression in the females in the PCR analysis. Testing the genes with gDNA showed the presence of all 34 genes selected in both genders. This indicates that the selected highly expressed sex bias genes are genomically present in both genders of *E. marinus*. However, further testing using a greater number of genes may reveal a sex specific gene. Despite the extensive differences in sex, in most species the male and female genomes may only have relatively few genes specific to one gender such as genes found on the Y chromosome in mammals. Therefore, phenotypic variation in sex could largely result from differences in patterns of gene expression rather than gene exclusivity (Connalion and Knowles, 2005, Rinn and Snyder, 2005). This investigation has highlighted a large amount of genes that are sexually dimorphic in their expression and several markers for sex specific gene expression, although identifying genomic markers was unsuccessful.

One explanation of why detecting a sex marker has been so challenging is that the genetic sex does not correlate with the sex phenotype presented. This is a potential scenario because experimental reversal breeding experiments have shown that a genetic female can become a fully functional phenotypic male in crustaceans (Malencha, 1992, Suzuki et al. 1999). Whether this occurs in natural populations of *E. marinus* is open to question, although genetic male isopods infected with *Wolbachia* function as neo-females (phenotypically are female but have a male genetic makeup). The consequence could be that the female and male libraries that were used in majority of these investigations could contain both male and female genetic material, despite the fact that DNA was extracted from a single gender on the basis of morphology. Although, a marker for the phenotypic sex may have been achieved by these methods, further analysis of *E. marinus* populations that are not influenced by sex distorting parasites or ESD should be considered.

Ultimately, several molecular techniques were adopted and a number of genes that were expressed only in males were identified, although none were genomically sex specific. The study did identify AFLP analysis as the technique most likely to establish the presence of sex specific genomic regions in *E. marinus* and provided some insights into the future development of these methodologies. If GSD is present in *E. marinus*, then further investigations will be required to fully attempt to identify genomic differences. This would provide an opportunity to understand how environmental and parasitic factors can influence crustacean GSD, as well as explore the mechanisms behind sexual differentiation disruption observed in cases of *E. marinus* such as intersexuality.

7. General discussion

The overall aim of this investigation was to increase our knowledge of the general population dynamics of *E. marinus*, with a particular focus on attempting to elucidate the mechanisms of sex determination in this ubiquitous marine amphipod. In crustaceans, sex can be determined environmentally, via parasitic infection, or genetically, however, it is possible that in any given species, that all three factors are involved. Sex determination in *Echinogammarus marinus* (Leach, 1815), has been previously linked with feminising parasites (Ford et al. 2006). Although, prior to this study little was known about the extent to which the other parameters that could be involved.

Firstly, an *E. marinus* population was investigated over a two year period (2009-2011) to assess the population dynamics (chapter 2). During this study two parasites with contrasting life histories were also monitored and their seasonal prevalence analysed into relation to their host population dynamics (chapter 2). One particular parasite, a trematode belonging to the Microphallidae family was revealed as a potential new species and its biology and phylogenetics were investigated in chapter 3. The two *E. marinus* populations studied, Langstone Harbour, Portsmouth and Inverkeithing, Scotland appear to be governed by two different sex determining mechanisms. Seasonal sex ratio data from the field and subsequent laboratory findings presented in chapter 4 indicated that *E. marinus* possesses environmental sex determination (ESD) in a population that is not influenced by sex distorters (Langstone Harbour). The study reported in Chapter 5 investigated parasite transmission of two potential sex distorters in an *E. marinus* population with high female bias and intersexuality (Inverkeithing). Vertical transmission of a Paramyxean sp. was shown for the first time in an amphipod host. This has questioned *D. dubenum* as a feminiser and has highlighted another parasite candidate for *E. marinus* sex distortion. Despite extensive analysis (chapter 6), we still cannot determine the presence of genetic sex determination (GSD) in this species. This project's findings have suggested several future avenues of research which will be discussed in this chapter.

A monthly field study was conducted over a two year period and established that although a natural population of *E. marinus* from Langstone Harbour (Portsmouth, UK) breeds throughout the year, it does display some seasonality in the reproduction, with peaks in the spring and summer. The study also found that although there was evidence for a seasonally altered sex ratio, with swings that ranged from 35.5 % to 71.4 % males, the population had a 1:1 sex ratio over the entire sampling period. In addition, the field study confirmed that the Langstone harbour population had no sex distorting parasites, although, two parasite groups, one microsporidia (*D. berillonum*) and trematode (Microphallidae sp.), were found to infect the population. Temporal changes in these parasite groups were recorded and provided an insight in their seasonal prevalences with *D. berillonum* showing a consistent prevalence level. This is in contrast to the levels observed for the trematode, which peaked and crashed in a more dramatic fashion, a pattern most likely associated with its life cycle strategy. *D. berillonum* has been verified as a vertical transmitter; although due to the non sex bias infection prevalence and the lack of any female bias in the broods of infected mothers indicates that the species is not a feminiser in *E. marinus*. This is in conjunction with Terry et al. (2004) that also found *D. berillonum* not to have sex bias infection prevalence in several host populations.

The prevalence of the trematode parasite in *E. marinus* populations was found to be very high (70%) at certain times of the year and a clear correlation was observed between host abundance and parasites numbers. Trematodes have been known to be very influential in amphipod populations (Poulin and Mouritsen, 2006). For example, Damsgaard et al., (2005) found that a population of corophium amphipods infected with trematodes is prone to collapse. For this reason, the biology of the trematode was investigated (chapter 3) with respect to its effect on *E. marinus* and a phylogenetic analysis was conducted to determine the species of parasite. The sequencing of the gene for rDNA of the trematode found in the Langstone Harbour population revealed that the trematode belongs to the Microphallidae family and is most likely a new species. The same species of trematode was also found in the Scottish Loch Fleet *E. marinus* population, with the Inverkeithing population

presenting infection by a different trematode belonging to the Opecoelidae family. The trematode found in the Portsmouth population encysts within the brain and shows a similar capacity for host behavioural manipulation as that induced by the trematode *Microphallus papillorobustus* in the gammarid *Gammarus insensibilis* (Helluy, 1982, 1984). Phylogenetic analysis indicated that the two trematodes were not the same species, as other microphallids had closer identity. The behavioural manipulation induced by the parasite can lead to higher predation risk for the amphipod. The gene expression study highlighted possible neuro- modulatory genes being altered with a putative serotonin receptor 1A like gene showing up to a 7.2 fold upregulation and the inebriated 2 neurotransmitter like gene presented a 6.7 fold down regulation in the infected group. The seasonal prevalence of the parasite showed significant correlation with host abundance. This, in combination indicates this trematode species has the capability to highly influence population dynamics of *E. marinus*.

Previous studies have demonstrated that ESD exists in some populations of amphipods (Dunn et al. 2005). Chapter 4 details an investigation to determine if ESD occurs in *E. marinus*. Field study and laboratory breeding experiments were conducted to determine the influence of photoperiod on sex determination. This was achieved by re-analysing sex ratio data from chapter 2 and a series of laboratory breeding experiments under different light regimes. Over the 2 year field study, males dominated during August to November whilst female biased populations were observed during April to July. A significant linear relationship was observed between photoperiods and sex ratios from the field data. Under laboratory conditions photoperiod was also shown to be an influential factor in sex determination, with a male bias over a long day photo regime (61.5% male broods) and a female bias over a short day photoperiod regime (43.5% male broods). These photoperiod influences in sex determination correlate with findings in the brackish water amphipod, *Gammarus duebeni* (Dunn et al. 2005). Findings suggest that there is some level of ESD present within *E. marinus*, suggesting considerable plasticity in the sex differentiation pathway.

The presence of ESD in other *E. marinus* populations remains uncertain; other studies of *E. marinus* populations have shown no indication in ESD, possibly due to geographical location (Maranhao et al., 2001) or other sex determining factors, such as sex distorting parasites, not being considered (Vlasbloom, 1969). The presence of ESD in other *E. marinus* populations should be considered but preferably examining populations in varied environments that have seasonal reproduction and are not infected by sex distorting parasites. The possibility of a second cue being present, such as temperature, should also be examined as Dunn et al. (2005) found that some *G. duebenum* populations were influenced by the interaction of temperature and light. Whether *E. marinus* populations that do suffer from parasite induced sex distortion still have an underlying ESD could be examined. The *E. marinus* population at Inverkeithing would be an obvious choice and could be performed by a field and laboratory sex ratio study of uninfected individuals.

Previous studies have shown that feminising parasites can alter the sex ratios of amphipod populations (Ford et al., 2006, Ironside et al., 2003, Mautner et al., 2007, Terry et al., 2004, 2007). In *E. marinus* studies have found links between intersexuality and female biased sex ratios with parasites thought to be feminising (Ford et al., 2006). This study (chapter 5) investigated the transmission of these parasites, in particular the *E. marinus* population at Inverkeithing. Infection prevalence in the sexual phenotypes suggests that *E. marinus* is influenced by PSD and validates past work. The *E. marinus* Inverkeithing population has high female bias, high levels of intersexuality and is infected by two potential sex distorting parasites that have high infection rates in the female and intersex phenoptypes. Although the study did not determine whether the paramyxean or the microsporidian (*D. duebenum*) was responsible for this sex distortion, the study found significantly high co-infection prevalence, indicating a possible hitchhiking strategy by one of the parasites. The paramyxean only infections were more prominent and the parasite presented higher levels of vertical transmission compared with *D. duebenum*. The paramyxean showed a reduction in transmission efficiency when co-infected with *D. duebenum*, indicating possible regulatory effects when the two parasites infect one individual. A greater understanding of the association between microsporidia and Paramyxea is required. Past studies highlighting microsporidian influences on sex

determination need to be reconsidered and screens for paramyxeans performed. This would identify if the co-prevalence of these vertical transmitters in the Inverkeithing population is an isolated or common case. If paramyxean presence is not common it would substantiate the evidence from past studies on microsporidians, in particular *D. duebenum* being a feminising agent.

Experimental horizontal transfer was carried out by infecting uninfected individuals with co-infected tissue. This showed the paramyxean can successfully transfer while *D. duebenum* showed no signs of transmission in *E. marinus*. This is either because *D. duebenum* is incapable of experimental horizontal transmission and only transmits vertically or insufficient conditions in the experimental design did not allow for *D. duebenum* transmission. For example, within the co-infected tissue used, the paramyxean spores could potentially be hardier and therefore transferred into the uninfected individual successfully, whereas *D. duebenum* spores may have not been viable when inoculated. Although, it is worth noting that similar experiments have been successful in artificially infecting amphipods with microsporidia (Dunn and Rigaud, 1998). In addition, the paramyxean infection that was horizontally transmitted to an *E. marinus* female was then passed vertically to the brood, opening up possibilities for further investigation. Females showed greater infection burden than males following experimental horizontal infection, indicating a sex bias in their susceptibility, although further study is required to validate these findings. Future work could determine whether the paramyxean has feminising capacity by artificially infecting females and studying the brood sex ratios. Further investigations could also be conducted to verify whether *D. duebenum* and *D. berillonum* can horizontally transmit under laboratory conditions. This would give us a greater understanding of whether less successful vertical transmitters employ both transmission pathways and verify whether *D. duebenum* cannot be artificially transferred horizontally. In addition, the question of whether vertical transmitters can horizontally transmit naturally could be addressed by conducting co-inhabitation experiments, thereby mimicking field conditions.

A series of genomic techniques were employed to determine whether a sex specific region of DNA could be found in *E. marinus* and thus add weight to the evidence that amphipods, along with other crustaceans have genetic sex determination (chapter 6). The attempt to determine if genetic sex determination (GSD) is present in *E. marinus* was not successful and the study did not identify a genomic sex marker. However, future examination for the presence of GSD in *E. marinus* is required before conclusive statements can be made. The study eliminated androgenic gland (AG) manipulation as a tool for determining the heterogametic sex in *E. marinus*, as it was problematic, time consuming and possibly not achievable. Animals that underwent the AG manipulation and the artificial parasite infection experiment showed no signs of intersexuality or sex reversal, suggesting that it may not be possible to experimentally sex reverse an *E. marinus* adult. Transcriptomic mining for sex exclusive genes also showed little promise with PCR analysis indicating the presence of all 34 genes selected in both genders. Phenotypic variation in sex could be a result of differences in patterns of gene expression rather than the exclusive presence of a sex determining DNA region in one gender. Male and females of any species does not necessarily need distinct genomes to possess variation. It has been suggested that differences in gene expression is highly important in gonochoristic animals that possess non genetic sex determination such as ESD and PSD (Small et al. 2009). Therefore in *E. marinus*, rather than having large genome differences between sexes, sex may rely on sexual dimorphism at the level of transcriptomic variation. This would allow for greater sexual plasticity in the sex phenotypes as all genes required to be male or female would be present, whilst other sex determining mechanisms govern.

AFLP analysis has been successfully applied to identify sex specific genomic regions in crustaceans (Ventura et al. 2011, Staelens et al. 2007, Zhang et al., 2007). The AFLP analysis appeared most likely to find genomic differences between *E. marinus* sex, with obvious differences in the band patterns seen in the highly resolved polyacrylamide gels. AFLP banding patterns indicated genetic variation among the sexes and with methodology adjustments described in chapter 5 a sex marker might possibly be isolated. The ISSR and RAPD analyses revealed non-sex specific individual variation, indicating these techniques could be useful tools in population

genetic studies, as well as investigating levels of genetic variation in populations that suffer from environmental contamination. Selective subtraction hybridisation (SSH) is an alternative technique that could be employed to isolate sex specific DNA regions in *E. marinus*. The fundamental aim of genomic subtractive hybridisation is to distinguish and isolate DNA sequences that are present in a tester sample (i.e. females), however, absent from the driver sample (i.e. males) and vice versa (Straus and Ausubel, 1990). This protocol in conjunction with high throughput sequencing of the gender specific library could be a powerful tool in highlighting genomic differences in E. *marinus* as well as indicating the heterogametic sex. Although, this is currently an expensive method, especially considering that the presence of genetic variation between the sexes is not certain. Overall, this study highlighted several genes exclusively expressed in males by mining transcriptomic data and provided some insight into the future development of AFLP analysis in *E. marinus*.

During this study it has been determined that an *E. marinus* population at temperate latitudes has a seasonal breeding pattern, with population growth and decline closely related to environmental parameters (e.g. temperature) and parasites (e.g. trematodes), respectively. A new species of trematode parasite has been identified that demonstrates clear capacity for behavioural changes in its host. These behavioural changes have been linked to changes at the level of gene expression suggesting the modulation of neuronal genes in the infected individuals. This represents the first study to record such changes in the serotonin pathways of parasite infected amphipods. ESD has been shown in *E. marinus* for the first time and the role played by parasites in the sex determination of *E. marinus* is now better understood, with transmission pathways and efficiency having been established. Despite the range of genomic techniques employed, the attempt to determine genomic sexual determination in *E. marinus* was less successful. However, considering the preliminary nature of the work, this study has provided insight for future directions. Several key genes involved in sexual differentiation that presented sex exclusivity in their expression were identified. In addition, crucial method development was performed that will allow future investigations of genetic variation in *E. marinus*. The importance of *E. marinus* as a key ecological model species is growing (Dick et al. 2005, Egilsdottir et al., 2009, Ford et al. 2003, 2005, 2006, Guler and Ford, 2010,

Maranhao et al., 2001, Maranhao and Marques, 2003, Martins et al., 2009, Pastorinho et al., 2011, Yang et al., 2008, 2011, Short et al., 2012b). The transcriptome of the *E. marinus* has now been sequenced and along with population models, will enable links between genome and population ecology. With such large investments in *E. marinus* as a model it is crucial that basic biological questions and gaps in the field are addressed. Consequently, the data presented within this thesis will aid in the study of *E. marinus* and other crustaceans from genetic to population level effects.

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9. Appendices