

**The Epigenetic Effect of Trematode Infection  
on the Snail Host *Zeacumantus subcarinatus***

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## Abstract

Parasites are able to modify their host's morphology and physiology to increase their own fitness and transmission success; this phenomenon is known as parasite-induced host manipulation (PIHM). Though PIHM is widely observed in many lineages, the exact mechanism by which parasites elicit these effects is yet to be determined. However, parasites may induce these host modifications by causing changes at the DNA level by causing epigenetic changes. Epigenetics encompasses environmentally-induced heritable changes in the phenotype through changes in gene expression patterns without the primary nucleotide sequence being altered. The changes in gene expression occur due modifications at both the DNA and histone level which act to either activate or silence gene expression; various hypotheses exist about the mechanisms behind these changes in gene expression. These epigenetic changes often lead to dramatic biological outcomes due to their important roles as gene regulators. Given that parasitic infection is a major environmental factor on the host, and epigenetic changes are highly reactive to such environmental factors, it is very plausible that parasites may be exploiting this system to induce the PIHM phenotypes observed. Thus, the current study investigated whether epigenetic differences were present in the mud snail *Zeacumantus subcarinatus* when infected with the trematodes *Philophthalmus* sp. and *Maritrema novaezealandensis* compared to uninfected conspecifics. Differences in epigenetic modifications between infected and uninfected snails were investigated at both the DNA and histone level using enzyme-linked immunosorbent assay (ELISA) to determine global DNA methylation levels and mass spectrometry (MS) to determine the patterns of histone modification in infected versus uninfected individuals. No significant differences in mean 5-methylcytosine levels were detected between uninfected *Z. subcarinatus* snails and those infected with either *Philophthalmus* sp. or *M. novaezealandensis*. Similarly, no overall differences were found in the pattern of global histone post-translational modifications between uninfected *Z. subcarinatus* snails and those infected with either *Philophthalmus* sp. or *M. novaezealandensis*. Therefore, the current study determined that parasitic infection does not broadly affect host epigenetics. These findings fit with the current knowledge in the field which finds that epigenetic changes on such broad levels often lead to disease, as these epigenetic marks are important gene regulators. Any parasite-induced epigenetic changes in their host are likely to be targeted at the level of DNA methylation patterning and changes at the individual quantity of each histone PTM, which are known to exert strong biological effects, therefore future studies should be focused at such a level.

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# Table of Contents

Abstract .....	ii
Acknowledgements .....	iii
Table of Contents .....	iv
List of Tables .....	vi
List of Figures .....	vii
List of Abbreviations .....	viii
1 General Introduction .....	1
Parasites affecting Host Neuromodulators and Hormones .....	3
Epigenetic Modifications .....	6
Examples of Naturally-occurring Epimutations .....	7
Single-celled Parasite affecting Host's Epigenetic Profile .....	9
Study System .....	11
Aims of the Study .....	16
2 General Methods .....	17
Animal Collection .....	18
Tissue Harvesting .....	18
3 DNA Methylation Analysis .....	20
3.1 Introduction .....	21
DNA Methylation Establishment .....	23
Examples of Environmental Stimuli inducing changes in DNA Methylation .....	24
Aims .....	25
3.2 Methods .....	26
DNA Extraction .....	26
DNA Quantification .....	27
Ethanol Precipitation .....	27
Enzyme-Linked Immunosorbent Assay (ELISA) .....	28
Calculation of Relative DNA Methylation .....	30
Statistical Analyses .....	30
3.3 Results .....	31
3.4 Discussion .....	32
DNA methylation patterning in Cancer Epigenetics .....	32
DNA Methylation in Invertebrates .....	33

Conclusions .....	34
4 Analysis of Histone Post-translational Modifications.....	35
4.1 Introduction .....	36
Histone Modification mechanisms .....	38
Examples of Environmental Stimuli inducing Histone Epigenetic changes.....	40
4.2 Methods.....	43
Shotgun Proteomics for Protein Identification .....	43
Histone Extraction .....	43
Sodium Dodecyl Sulfate ó Polyacrylamide Gel Electrophoresis (SDS-PAGE) .....	44
Making the Gel .....	45
Running the Gel.....	46
Tryptic Digestion.....	47
Ionisation of Tryptic peptides using LC-MS/MS .....	48
Tandem Mass Spectrometry: Instrument Setting for the LTQ-Orbitrap .....	49
Data Analysis.....	50
Criteria of Data for Inclusion .....	51
4.3 Results .....	53
4.4 Discussion .....	63
Histone PTM Quantification and Patterns .....	63
Histone Code Hypothesis.....	64
Expansion upon Histone Code Theory .....	64
Conclusions .....	65
5 General Discussion .....	66
Shell formation .....	67
Parasites affecting Gene Expression.....	68
Recommendations for future directions.....	70
Conclusions .....	71
6 Additional Methodological Details.....	72
6.1 Gel Recipes.....	73
6.2 Buffer Recipes.....	74
6.3 Stain Recipe.....	74
7 References .....	75

## List of Tables

Table 1. Examples of histone modifications and their likely function .....	37
Table 2. Detected post-translational modifications on histone H2A .....	53
Table 3. Detected post-translational modifications on histone H2B Gonadal .....	54
Table 4. Detected post-translational modifications on histone H3 .....	57
Table 5. Detected post-translational modifications on histone H4 .....	61

## List of Figures

Figure 1. Life cycle of <i>Philophthalmus</i> sp. (Digenea: Philophthalmidae) trematode. ....	13
Figure 2. Life cycle of the trematode <i>Maritrema novaezealandensis</i> . ....	14
Figure 3. Percentage overall DNA methylation for whole <i>Zeacumantus subcarinatus</i> individual snails which are uninfected (Ux), infected with <i>Philophthalmus</i> sp. (Px), and infected with <i>Maritrema novaezealandensis</i> (Mx). ....	31
Figure 4. How histone acetylation and deacetylation affect chromatin structure. ....	40
Figure 5. Diagramatic representation of the gel pouring and sample loading formation. ....	46
Figure 6. Colour configuration of modifications on amino acids. ....	52
Figure 7. Comparison of H2B Gonadal sequences matched by MASCOT program between five samples of <i>Zeacumantus subcarinatus</i> snails. ....	55
Figure 8. Comparison of <i>Aplysia californica</i> sequences matched by MASCOT program between histone H3 samples from five samples of <i>Zeacumantus subcarinatus</i> snails. ....	59
Figure 9. Comparison of <i>Mytilus edulis</i> sequences matched by MASCOT program between histone H4 samples from five samples of <i>Zeacumantus subcarinatus</i> snails. ....	62

## List of Abbreviations

5-hmC	5-hydroxymethylcytosine
5-HT	Serotonin
5-mC	5-methylcytosine
ANOVA	Analysis of variance
C	Carboxyamidomethyl cysteine
CID	Collision-induced dissociation
DA	Dopamine
DNMT	DNA methyltransferase
DTT	Dithiothreitol
EDC	Endocrine-disrupting chemical
E-value	Expected value
GBP	Growth-blocking peptide
HPLC	High-performance liquid chromatography
JH	Juvenile hormone
K	Lysine
LC	Liquid chromatography
LG-ABN	Maternal licking and grooming and arched-back nursing
M	Oxidation
M	<i>Maritrema novaezealandensis</i> infected <i>Zeacumantus subcarinatus</i> snail
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NQ	Deamidation
N-terminal	Amino acid terminal
P	<i>Philophthalmus</i> sp. infected <i>Zeacumantus subcarinatus</i> snail

PCR	Polymerase chain reaction
PIHM	Parasite-induced host manipulation
PTM	Post-translational modification
R	Arginine
RFU	Relative fluorescence units
RP-HPLC	Reversed-phase high-performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulphate ó polyacrylamide gel electrophoresis
TET proteins	Ten-eleven translocation proteins
U/UN	Uninfected <i>Zeacumantus subcarinatus</i> snail

# **1 General Introduction**

Many parasite lineages have complex lifecycles involving more than one host, and eventually have to transmit to a new host to continue their development (Choisy *et al.*, 2003). Successful transmission is, however, very improbable, as parasites have a high chance of transmitting to an inappropriate host or fail to encounter any potential hosts at all. Consequently, these selection pressures have caused parasites to evolve various traits to increase the likelihood of lifecycle completion. One of the earliest traits identified, and possibly the most observable, is high fecundity (Combes, 1991). However, any adaptation which increases transmission success plays an equally important role, as it fundamentally enhances parasite fitness (Combes, 1991). Possibly the most intriguing adaptation is parasite-induced host manipulation (PIHM), where parasites induce phenotypic changes in the host in ways which increase transmission success. PIHM can manifest in both behavioural and physical changes in the host; parasites have been documented to affect their host's offspring care (Libersat *et al.*, 2009), cause host paralysis (Libersat *et al.*, 2009) and colour change (Yanoviak *et al.*, 2008), affect social behaviour (Klein, 2003), and even drive their hosts to suicide (Libersat *et al.*, 2009), all to the benefit of the parasite.

A particularly interesting example of this transmission strategy is that of the parasitic protozoan *Toxoplasma gondii* which infects rats as the intermediate hosts, and subsequently cats as the definitive host. Infected rats are significantly less averse to cat odours, and even seem to show a preference for areas with signs of cat presence (Berdoy *et al.*, 2000). Interestingly, this alteration by *T. gondii* infection seems to be confined to the predator's odour, as both infected and uninfected rats behave similarly in regards to their own odour and that of other non-feline animals (Berdoy *et al.*, 2000). *Toxoplasma gondii* thus appears to manipulate a natural defence mechanism in rats, thereby aiding parasitic transmission via predation, to the feline definitive host. This implies *T. gondii* specifically affects the cognitive perception of the host to the natural predator, rather than causing a gross impairment of olfactory faculties (Berdoy *et al.*, 2000). *Toxoplasma gondii* is a very well-studied case of PIHM acting on the intermediate host's behaviour; but, there are many more studies in which documented PIHM leads to morphological changes in the host. For example, one of the most striking examples of PIHM inducing both morphological and behavioural changes is that of the nematode infection of the tropical canopy ant, *Cephalotes atratus* (Yanoviak *et al.*, 2008). The induced changes in the host are dramatic, causing the infected ants to convincingly mimic *Hyeronima alchorneoides* fruits, which are eaten by the definitive bird hosts of the parasitic nematodes; this manipulation is necessary for transmission, as the definitive bird host is strictly frugivorous and thus does not normally eat the ants. In this system, developing

nematode larvae are found exclusively in the ant's gaster, the posterior portion of the abdomen, where they alter the pigment density of that region, causing it to become a translucent amber colour. This, in combination with the yellowish nematode eggs held inside, creates the bright red fruit-like appearance of the gaster, mimicking that of the fruit, in contrast to the jet-black gaster of uninfected ants. Remarkably, the intensity of the red colour is positively correlated with the timing of nematode embryo maturation, and reflectance profiles show close similarities between infected ant gasters and mature *H. alchorneoides* fruits. Furthermore, the post petiole-gaster junction is 93% weaker in infected individuals, which, in addition to the natural grip that ants have on twigs, means that the parasite-laden gaster can be easily plucked by the definitive host. Experiments testing dummy gasters in the wild confirm the red colour is appealing to frugivorous birds, as gaster removal rates were significantly higher for infected- than for normal-coloured dummy gasters. The behavioural changes induced by infection include near-constant gaster flaging and a very erect gait. Additionally, those with the most intense red colouration were found to be the least aggressive, produced no alarm pheromones when disturbed, and had the most sluggish and unstable gait. Collectively, the bright-red appearance of the gaster, the reduced alarm defences, the weakened gaster-post petiole junction, and the general sluggishness likely aids consumption of an infected gaster by a fooled frugivorous definitive host.

Due to the prevalence of PIHM cases and the often extreme ways in which host phenotypes are manipulated by their parasites (see Moore, 2002 and Poulin, 2010), PIHM has been of considerable interest to parasitologists. In particular, interest has centered on the mechanisms by which parasites achieve PIHM. These mechanisms will be explored by reviewing examples of parasites manipulating host neuromodulators and hormones to achieve phenotypic outcomes, followed by a postulation that epigenetics may be the mechanism driving these changes in host physiology. The important role of epigenetic influences on gene expression is discussed with references to well-known examples, and cases in host-parasite systems. Lastly, the study system used in the current study will be described with the specific aims set out.

### *Parasites affecting Host Neuromodulators and Hormones*

Classically, parasite-induced behavioural and physical alterations in the host have been hypothesised as the result of biochemical manipulation by the parasite. For instance, parasite-induced changes in brain monoamine activity levels are comparable to a chronic

stress response in some species (Øverli *et al.*, 2001), but appear to suppress the innate stress response in others (Shaw *et al.*, 2009). It is possible that these modifications lead to a change in host behaviour during times of stress which may be beneficial for the parasite, such as in the presence of a predatory definitive host (Shaw *et al.*, 2009).

However, the most common neurological systems studied have been the dopamine (DA) and serotonin (5-HT) pathways following infection. Changes in both DA and 5-HT levels often lead to changes in host social behaviour such as increased conspecific aggression and wounding (reviewed in: Klein, 2003), manipulations which are beneficial for single-host parasites to increase their transmission throughout their host population. Moreover, changes in DA levels have often been reported following infection (see: Klein, 2003, Shaw *et al.*, 2009, Rojas and Ojeda, 2005), with more pronounced changes associated with greater parasite density (Shaw *et al.*, 2009). Studies of parasitic manipulation of the 5-HT pathway have been more conclusive. For example, infection of the amphipod *Gammarus insensibilis* by the larval trematode *Microphallus papillorobustus* simultaneously affects the 5-HT levels in specific regions of the host brain and widely alters the integrity of the serotonergic neurons, causing stunting and deformation of various neurons (Helluy and Thomas, 2003). This parasite-induced disruption of the host's brain morphology and connectivity of the serotonergic neurons contribute to the altered responses to environmental stimuli exhibited by infected individuals, therefore making them more susceptible to predation by the definitive host. Given such findings, it is apparent that parasites are able to elicit definite effects on their host's neural biochemistry; likewise, parasites are also very well-known to hormonally manipulate their hosts, though not always to aid transmission.

Changes in host endocrinology following infection have become a popular research direction in the field of PIHM. Most of the literature in this field is focused on parasitoids, a type of parasite in which the larvae develop by feeding off the bodies of their hosts, but the rest of the life cycle is free-living (Godfray, 1993); the most studied parasitoids are wasps that use other insects as hosts. Though the host usually dies following the departure of the parasitoid, the parasitoid induces many dramatic changes in the living host during the course of infection. Broadly, parasitoid infection has been found to decrease the host's overall fecundity (Sait *et al.*, 1994), egg production and egg viability in a dose-dependent manner (Bjornstad *et al.*, 1998). However, most of the research into parasite-induced host hormone change has focused on the common developmental delay. This delay is caused by reducing the host feeding rate, thus impeding overall food consumption and decreasing host growth rate (Jones *et al.*, 1981, Beckage and Kanost, 1993, Sait *et al.*, 1994, Goulson and Gory, 1995,

Bjornstad *et al.*, 1998). Consequently, it takes longer for the host to reach the minimum critical size required to initiate metamorphosis to the next growth stage, extending the time the host remains in the larval stages (Beckage and Kanost, 1993). It is thought that this allows the parasitoid larvae, which are often located in the host's haemocoel, sufficient time to develop before the host develops in a way which hinders parasite emergence (Kyei-Poku and Kunimi, 1998); such as developing a tanned cuticle or encasing itself in a protective cocoon (Beckage and Kanost, 1993).

Parasitoids often retard larval development by increasing haemolymphic DA (Tanaka *et al.*, 1987, Noguchi and Hayakawa, 1996), however, the majority of studies of parasitoid-insect systems are focused on the changes in the juvenile hormone (JH) system and its associated hormones. JH is an important insect hormone responsible for maintaining larval structures during development as well as inhibiting metamorphosis (Ruppert and Barnes, 1994, Hickman *et al.*, 2007). JH's effects depend on concentration: High JH levels cause a larva to moult into a larger larva, lower JH levels cause a larva to moult into a pupa, and no JH in the system causes the pupa to develop into an adult (Ruppert and Barnes, 1994). Parasitoids appear acutely adapted to sense JH changes in the host, as the normal decrease of JH levels later in the instar stimulates the emergence of the parasite from the host (Beckage and Riddiford, 1982). Rather than just aiding the parasitoid to escape the host before it is unable to, parasitoid infection also significantly increases haemolymph JH levels and up-regulates JH production in the last instar larvae (Beckage and Riddiford, 1982), thereby delaying host development. Moreover, parasitised larvae also display low levels of haemolymph JH esterase (Beckage and Riddiford, 1982), one of the principal degraders of JH which lowers JH levels in preparation for pupation (Hammock, 1985). These parasite-induced increases of JH may also help ensure the parasite's access to host resources by preventing pupation-related physiological changes, such as fat body synthesis of storage proteins prior to metamorphosis (Beckage and Riddiford, 1982). Additionally, the hormone growth blocking peptide (GBP) is also exploited by parasitoids to delay host development (Hayakawa, 1995). GBP is naturally-occurring in lepidopteran larvae and has a similar role to JH: it is believed to prevent premature pupation with concentrations varying according to the developmental stage of the insect (Hayakawa, 1995). Parasitoids are thought to reactivate the GBP gene in mature larvae, causing synthesis outside of normal developmental need, thus inhibiting larval development (Hayakawa and Yasuhara, 1993, Hayakawa, 1995). It appears that GBP prolongs the last instar stage by inhibiting JH activity (Hayakawa and Yasuhara, 1993).

In contrast to the above examples, some parasitoids induce precocious development of the host, such as triggering premature cocoon spinning, yet not allowing the host to pupate.

This manipulation appears to benefit the emerging parasitoid, as it allows the parasite to emerge and develop in a protected environment (Jones *et al.*, 1981). Host manipulation achieved by hormonal alteration appear to be both wide-spread and effective among parasitoids, perhaps due to their phylogenetic proximity with the host (both hosts and parasitoids are insects), meaning they likely share common biochemical pathways that the parasitoid can exploit.

Nevertheless, the exact mechanism used by parasites to elicit these and other PIHMs has yet to be determined. Although many studies find definitive evidence that parasites induce hormonal and neuromodulator changes, more profound, wide-reaching mechanisms may be involved. Indeed, it may be parasite-induced epigenetic modifications leading to biochemical, morphological and behavioural changes, that leads to observable phenotypic changes.

### *Epigenetic Modifications*

Conventionally, the field of evolutionary ecology has regarded random genetic mutation as the solitary source of new genetic variation within populations (Maynard Smith, 1998). Maynard Smith (1998) describes that the four main evolutionary forces: mutation, random genetic drift, natural selection, and gene flow, help define evolutionary ecology. These act within and among populations to cause micro-evolutionary change which may eventually lead to significant macro-evolutionary patterns, arising in the longer term from the collective action of the four forces.

However, this view of genetics is being reformed by the addition of another dimension: epigenetics. Epigenetics encompasses environmentally-induced heritable changes in phenotype through changes in gene expression patterns without the primary nucleotide sequence being altered (Richards, 2006, Jablonka and Lamb, 2005, Bird, 2007). This is achieved through methyl and acetyl groups covalently bonding to the DNA sequence itself or to histone tails which affect its associated DNA, thereby affecting gene expression (Richards, 2006, Cooney, 2007). Currently, there are three defined epigenetic mechanisms: (1) DNA methylation which generally occurs at the base cytosine and sequesters DNA, making it less transcribable (this will be detailed in Chapter 3; Jablonka and Lamb, 2005, Richards, 2006); (2) histone amino acid acetylation and methylation which remodels chromatin structure and affects how readily the associated genes can be transcribed; and finally, (3) RNA interference causing gene silencing, where normal gene sequences are mistakenly identified as intragenomic parasitic sequences and hence being subject to suppression by the cellular

immune system (Jablonka and Lamb, 2005). Additionally, cells are known to be independently capable of passing on their epigenome (the chromatin structure and DNA methylation on the genome (Jablonka and Lamb, 2005, Cooney, 2007)) to their daughter cells during mitosis (Jablonka and Lamb, 2005). Many argue that these modifications must be heritable to be defined as epigenetic in nature (reviewed in: Richards, 2006), though epigenetic heritability is not examined in the current study.

Epigenetic modifications are evolutionarily significant as they act as a force on which external and/or internal, biotic and/or abiotic, environmental factors can shape the resulting phenotype by modulating the genetic information expressed, and it is the phenotype upon which natural selection has its effects. Thus epigenetic modifications have the power to influence evolution. Epigenetics therefore adds another dimension for evolutionary forces to act upon by allowing natural selection to influence the genotype within one generation.

#### *Examples of Naturally-occurring Epimutations*

There are now many examples of epigenetic modifications manifesting in dramatic phenotypic changes in both animals and plants. Perhaps the best-known model of epigenetic control and inheritance is the regulation of coat pattern in mice and its associated effects on body mass. The *agouti* gene in mice regulates the production of the wild-type coat colour by causing the hair follicle melanocytes to switch from synthesising black to yellow melanin (reviewed in Morgan *et al.*, 1999). This gene can be subject to the viable yellow (*A<sup>vy</sup>*) mutation which produces a completely yellow coat colour by inducing ectopic *agouti* gene expression; gene expression normally only being expressed in the hair follicles (reviewed in Morgan *et al.*, 1999, Waterland *et al.*, 2008). This phenotypic outcome varies according to the methylation level of the *A<sup>vy</sup>* mutated *agouti* promoter: higher levels of methylation result in gene silencing, producing black coats while yellow coats result from lower methylation and increased gene activity; proportional levels of coat mottling according to methylation level between these extremes (Morgan *et al.*, 1999). The methylation level, in turn, is correlated to the levels of maternal methyl supplementation during embryogenesis, specifically, the level of methyl donors (e.g. folate, choline, betaine and vitamin B12) in the maternal diet during pregnancy (Morgan *et al.*, 1999, Cropley *et al.*, 2006). Though this epigenetic system has been thought of as a clear model for the heritability of a stable epigenetic state through the germline without further exposure to the epigenetic agent (Cropley *et al.*, 2006), recent evidence eludes otherwise. It is now thought that rather than inducing new methylation to establish, the persistence of this phenotype through generations is due to an incomplete

clearance of the methylation during embryonic reprogramming (a phenomenon during embryogenesis where parental epigenetic marks are removed and new specific epigenetic patterns are established), with the maternal epigenetic mark being more resilient to reprogramming than its paternal counterpart (Morgan *et al.*, 1999, Blewitt *et al.*, 2006, Waterland *et al.*, 2007). Nevertheless, this remains an example of an epigenetic mark being obtained in one generation and affecting the phenotypes of successive generations.

Epigenetics affects not only physical traits but also animal behaviour, such as mate choice (Crews *et al.*, 2007). Environmental contamination by endocrine-disrupting chemicals (EDCs) has been shown to affect both the attractiveness of the selected individual as well as the perception of the selecting individual three generations after the initial exposure (Crews *et al.*, 2007). This effect is sex-specific, with third generation females showing discrimination against EDC-exposed males, whereas exposed males do not demonstrate such a preference. Thus, epigenetics has a significant and lasting effect on a major determining factor for evolution, sexual selection.

As mentioned, epigenetic effects are not limited to animals; they also modify the morphology of plants. The flowers of wild-type common toadflax, *Linaria vulgaris*, display dorso-ventral symmetry in both shape and colouration; however, a pleoric (regularity of structure occurring abnormally in normally irregular flowers) type of the flower also occurs, displaying radial symmetry. This phenotype results from aberrant heritable DNA methylation of the *Lcyc* gene which controls dorso-ventral asymmetry, causing silencing in the mutant plants (Cubas *et al.*, 1999).

Arguably the most fascinating epigenetic studies are those on genetically identical individuals, as they help negate the likelihood of cryptic genetic differences obscuring the link between epigenetic modifications and phenotypic outcomes. In humans, clones occur naturally as monozygotic (identical) twins, and it has been proposed that epigenetics represent the connection between an environmental factor and phenotypic differences, between such twins, which may have strong implications for disease research. Research into the epigenomes (DNA genomes including epigenetic methylations) of monozygotic twins found more dissimilarities arose in the pattern and overall methylation level between twins as they became older (Fraga *et al.*, 2005); with differences also arising among twins who had different lifestyles and had spent less of their lives together (reviewed in Wong *et al.*, 2005). These studies help clarify the underlying significant role of environmental factors in translating a common genotype into different phenotypes.

Though empirical evidence of the role of epigenetics in parasite-host manipulations is currently scarce, the few findings in the field are convincing and clear. For instance, the protozoan parasite causing malaria, *Plasmodium falciparum*, controls its own gene expression using epigenetic mechanisms to evade elimination by the host's immune system (Kyes *et al.*, 2001, Chookajorn *et al.*, 2007, Dzikowski and Deitsch, 2008). Infection is maintained by expressing a variable antigen, primarily the protein PfEMP1 encoded by the *var* gene, on the surface of the infected red blood cells. Each parasite has many variations of the *var* gene, so is able to express many different types of this protein, which help it escape detection and subsequent elimination by the host's immune system (Miller *et al.*, 1994). Recently transcribed *var* genes are silenced by methylation of lysine 9 at histone H3, allowing the marking and suppression of recently activated PfEMP1 variants, creating a molecular memory (Chookajorn *et al.*, 2007). This molecular memory ensures the set of PfEMP1 proteins in the cell is expressed in an orderly manner and each protein is expressed for a specified period of time then silenced. The silencing of PfEMP1 proteins lasts several cell cycles to prevent the premature exhaustion of the parasite's PfEMP1 repertoire (Chookajorn *et al.*, 2007). This aids in maintaining the infection, because by the time the host's immune system is able to recognise and eliminate protozoans expressing a specific PfEMP1 variant, other malaria parasites have already switched to expressing another variant to which the host immune system is naïve (Chookajorn *et al.*, 2007). This example provides evidence that parasites are able to use epigenetic mechanisms to interact with its host, though in this case the interaction is indirect.

#### *Single-celled Parasite affecting Host's Epigenetic Profile*

Studies of the bacterium *Wolbachia pipientis* provide the first clear evidence of parasite-induced epigenetic change in a host. *Wolbachia pipientis* is a wide-spread bacterial parasite among arthropods and is solely maternally inherited through incorporation into the female germline in the European leafhopper, *Zyginidia pullula* (Negri *et al.*, 2006, Negri *et al.*, 2009a, Negri *et al.*, 2009b). *Wolbachia pipientis*, therefore, has responded to this selection pressure by altering host reproductive anatomy to increase the proportion of females in the population, and hence the number of possible hosts. The bacterium feminises genetic males by causing them to develop into phenotypic intersexes; all-female *Z. pullula* populations can be in this way created. Though the specific mechanisms behind this are not yet fully ascertained, it is thought that *W. pipientis* achieves this feminisation by manipulating the expression of several genes involved in sex determination and development, thereby inducing

genetically male embryos to inherit the genetic imprint typical of females during development. Genomic imprinting is involved in sex-determination in insects by inducing the silencing of paternal genomes in lineages which use the haploid/diploid system (see Sánchez, 2008 for review). Specifically, the XO system used by *Z. pullula* often involves paternal X chromosome elimination upon fertilisation (see Sánchez, 2008 for review). Because the sperm carry 2X chromosomes and the oocyte carries 1X, the zygote initially has 3X chromosomes, one or two paternal X chromosomes are later eliminated depending on the intended sex of the embryo. Although it not explicitly stated, one may infer that because genomic *Z. pullula* males can be induced into developing into phenotypic females, the paternal X chromosomes are possibly suppressed rather than eliminated in this species, and it may be that suppression that *W. pipientis* is able to exploit. The intersex males develop either a typical ovariole histological structure or an altered ovary structure, causing them to develop eggs in place of sperm. Interestingly, the effect of *W. pipientis* on the phenotypic outcome of males appears to be density-dependent; specifically, the density of *W. pipientis* in feminised males bearing testes is four times lower than in infected females and feminised males with ovaries. Furthermore, it seems that *W. pipientis* must exceed a threshold density before it can properly manipulate genomic imprinting to successfully induce feminisation. These feminised males also appear to be sexually appealing to uninfected males, as couplings are often observed and their feminised reproductive organs have been found to be full of sperm; however, progeny were only occasionally produced. Normal males re-appeared in the population after the removal of *W. pipientis* from the females, validating the coupling of the feminising effect with the presence of the bacterium.

Thus, it would appear that *W. pipientis* indeed acts as an epigenetically-influencing environmental factor on its host *Z. pullula*, directly manipulating the genomic imprinting pattern of embryos to a degree where the sex is changed. *Wolbachia pipientis* hence plays a role as an environmental force which has the ability to induce epigenetic trans-generational changes, much like the case of  $A^{vy}$  mice. These findings show that the bacterium *W. pipientis* is an effective model for studying how parasitic animals can act as environmental factors which induce heritable epigenetic changes in host gene expression; thus increasing their own fitness by increasing transmission success. The next step in studying the role of epigenetics in host-parasite interactions and whether this may be behind PIHM, is to determine whether multi-cellular parasites can have similar epigenetic effects on their hosts.

Epigenetics is essentially defined as external stimuli inducing heritable changes in gene expression. Parasites are known to be a major environmental factor affecting host biology and thus are likely sufficient stimuli to induce epigenetic changes in the host.

Likewise, hosts may also use this system of genetic transcriptional control as a means to defend against parasitic infection. Parasites may be inducing the observed targeted manipulations in their hosts by exploiting the epigenetic system's reactivity to environmental stimuli. Though parasite-induced changes in gene expression appear to be a sufficiently far-reaching mechanism to explain the broad phenotypic manipulations seen in infected animals (Poulin and Thomas, 2008), this idea awaits further empirical evidence.

In order to investigate the possible mechanisms underlying morphological changes induced by parasitic infection, the interaction between a parasitic trematode and its gastropod first intermediate host was studied. Trematodes have complex life cycles involving multiple hosts and often castrate (destroy the gonads of) their first intermediate hosts (Lafferty, 1993, Sousa, 1983) allowing proliferation in the space where the gonads were. Host castration allows the resources originally designated to host reproduction to be plundered by the parasite for its own growth and reproduction (see McCarthy *et al.*, 2004). In permanently castrated hosts, changes which occur post-infection are not adaptive to the host, as they cannot be passed on to offspring and therefore cannot be subject to selection. Post-infection changes are therefore either: (1) a general host reaction to infection; (2) a side-effect of parasite exploitation of host resources; or (3) beneficial for the trematode. Moreover, in addition to competition between the host and parasite for host resources, there is also conflict over the utilization of space within the host's body; this conflict is more intense in hosts confined by an external shell (McCarthy *et al.*, 2004). It would therefore be beneficial for the trematode if shell growth was focused in the parasitized areas of the host, minimising the allocation of resources to host growth and allowing increased space for parasite growth (McCarthy *et al.*, 2004). Indeed, this has been found to be true: even when host shell size and overall volume is decreased by infection, the space accessible to the trematode is significantly greater for any given shell length (Lagrue *et al.*, 2007). Trematode parasites are known modifiers of host shell shape, though the mechanism behind this long-observed PIHM is yet to be discovered, and is the focus of the current study.

### *Study System*

The current study specifically focuses on a snail-trematode system, in which the mud snail, *Zeacumantus subcarinatus*, is infected with the trematodes *Philophthalmus* sp. (Martorelli *et al.*, 2008), *Maritrema novaezealandensis* (Martorelli *et al.*, 2004), and *Microphallus* sp. (Martorelli *et al.*, 2008). Although it is difficult to differentiate between *M. novaezealandensis* and *Microphallus* sp. morphologically, *Microphallus* sp. is extremely rare

in the study population (less than 2% prevalence; Martorelli *et al.*, 2008), and so infections which appeared to be either *M. novaezealandensis* or *Microphallus* sp. were deemed very likely to be *M. novaezealandensis* and were noted as such. As mentioned, trematodes have complex life cycles; in this system, *Z. subcarinatus* is the first intermediate host of the mentioned trematodes, these parasites can subsequently infect a second intermediate host (often a crustacean) or encyst on a hard surface, and complete the lifecycle in a waterfowl shorebird definitive host. In this system, the host, *Z. subcarinatus*, displays a wider shell base when infected with *Philophthalmus* sp. or another trematode which infects this snail, *Acanthoparyphium* sp. (Hay *et al.*, 2005). Although a single infection by *M. novaezealandensis* is not known to alter shell morphology, snails infected by this parasite were included in this study as a control, to deduce whether infection by a non-manipulating parasite cause changes in the host's epigenetic make-up. Interestingly, a single infection by *Acanthoparyphium* sp. can alter the shell shape as much as a double infection consisting of *M. novaezealandensis* and *Philophthalmus* sp. (Hay *et al.*, 2005). However, given the naturally low prevalence of *Acanthoparyphium* sp. (approximately 1-3% prevalence), the two more prevalent parasites *Philophthalmus* sp. (5% to 30% prevalence (Martorelli *et al.*, 2008)) and *M. novaezealandensis* (up to 80% prevalence (Martorelli *et al.*, 2004)) were used; for life cycle of *Philophthalmus* sp. see Figure 1 and for life cycle of *Maritrema novaezealandensis* see Figure 2. It appears that although all the trematodes that parasitise this snail have the common trait of castrating the host, they have differing effects on shell morphology (Hay *et al.*, 2005); i.e. the different trematode species are able to manipulate shell morphology to different degrees in a common host (Hay *et al.*, 2005). However, whether these changes are the result of changes to the host epigenome is to be determined.

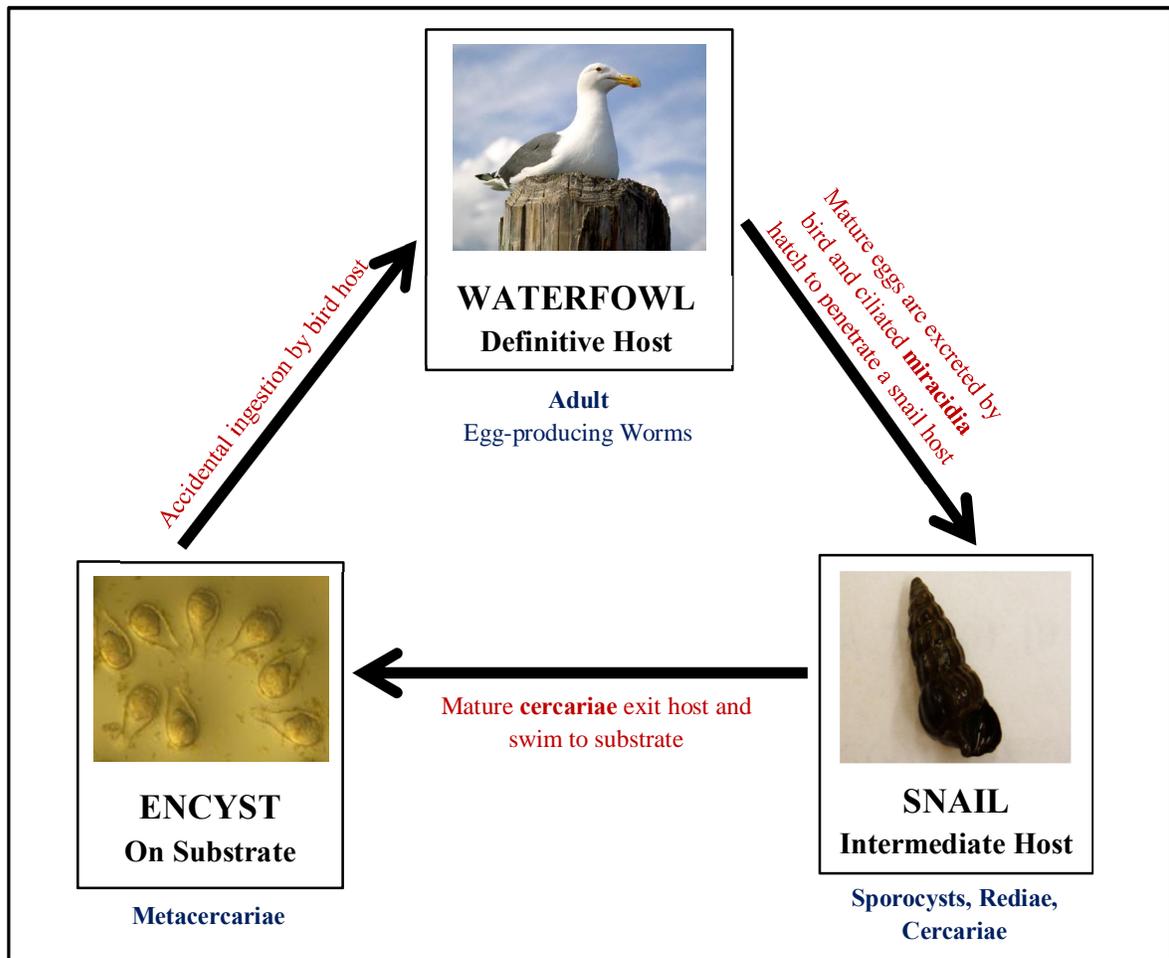


Figure 1. Life cycle of *Philophthalmus* sp. (Digenea: Philophthalmidae) trematode. Mature eggs are released by adult worms from the bird definitive host (Nollen and Kanev, 1995), ciliated miracidia hatch from these eggs immediately following contact with water and penetrate a snail intermediate host (Martorelli *et al.*, 2008). The miracidium injects a redia into the snail which can asexually produce more rediae (mobile larvae able to asexually produce more rediae or the infective cercariae larvae). Rediae act as dispersal units, spreading throughout the host tissue. Mature cercariae exit the snail and encyst on a hard substrate, which include food items where they are likely to be accidentally ingested by a bird definitive host, e.g. invertebrate exoskeletons, seaweed, mollusc shells (West, 1961, Nollen and Kanev, 1995). In the eyes of the bird definitive host, the metacercariae (encysted larval form of the parasite) develop into hermaphroditic egg-laying adults, thereby completing the life cycle (Nollen and Kanev, 1995). NB: cercariae can also directly infect the eyes. (Metacercariae photo courtesy of Fengyang Lei)

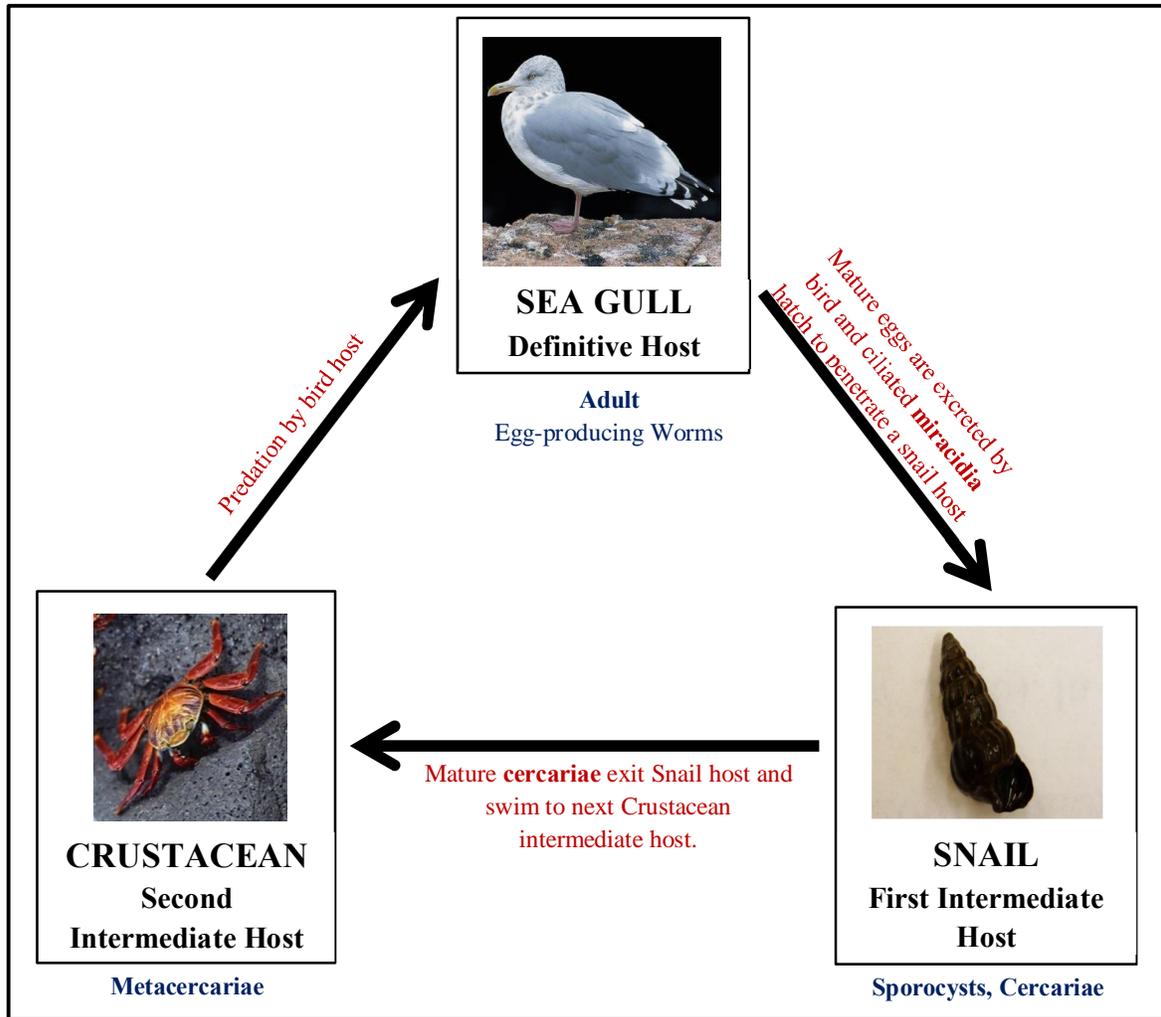


Figure 2. Life cycle of the trematode *Maritrema novaezealandensis*.

Eggs are released from the sea gull definitive host. The snail intermediate host becomes infected by accidental ingestion of the eggs. In the snail, the miracidium develops into a sporocyst which can asexually reproduce more sporocysts (an elongated sac able to asexually reproduce more sporocysts or the infective cercariae larvae). Mature cercariae exit the first intermediate host to infect a crustacean second intermediate host, mainly crab species but cercariae have also been found in amphipods. The cercariae encyst in the body cavity of the host and are known as metacercariae. These encysted metacercariae await natural predation of the crustacean host by the sea gull definitive host, where the parasite can become an egg-producing adult in the host's intestine, thereby completing the life cycle. Reviewed in (Martorelli *et al.*, 2004).

This type of parasite-induced morphological change is a relatively common occurrence in gastropod hosts. Parasitised snails display significantly more new growth (Krist, 2000), increased shell aperture length (Krist, 2000), and develop smaller and narrower (smaller width-to-length ratio) shells (Lagrue *et al.*, 2007) than their uninfected conspecifics. For instance, a recent study (McCarthy *et al.*, 2004) found that the trematode *Microphallus piriformes*, affects three aspects of its first intermediate host's (the rough periwinkle, *Littorina saxatilis*) shell: shell diameter, height and  $\alpha$ -angle (the angle of the top of the shell, the apex) are all modified. However, these parameters are each affected differently, not merely causing a uniform increase or decrease in shell size. Infection increases shell height in relation to diameter, and infected *L. saxatilis* tend to have a steeper  $\alpha$ -angle slope than uninfected conspecifics, resulting in shells with greater width-to-length ratios. It is postulated that these *L. saxatilis* were infected at a young age and grew shells of a larger volume, as the top whorls were elongated and grew at a smaller  $\alpha$ -angle, causing the shell to be taller.

In other snail-trematode associations infection, as well as changing shell dimensions, has been observed to affect snail shell spinyness, making them less likely to have and produce protective spines than uninfected snails (Levri *et al.*, 2005, Lagrue *et al.*, 2007). Interestingly, infected snails had smooth first (most recently secreted) whorls, but had spiny second or third whorls, suggesting that the degree of spinyness had changed since infection occurred (Levri *et al.*, 2005). Yet, it is unclear whether this reduction in spininess is due to the parasite directly suppressing the production, or whether the cost of parasitism causes spine production to become unsustainable (Lagrue *et al.*, 2007). Likewise, parasitic infection also has varying effects on shell thickness; some infected snails display shells which were generally thinner and more easily cracked than the shells of uninfected snails (McCarthy *et al.*, 2004), whereas other snails show no such changes following infection (Lagrue *et al.*, 2007). Again, the morphologically altered shells could be a by-product of parasitic leeching of host resources, resulting in lowered quality of the shell (McCarthy *et al.*, 2004).

Conclusively, trematode parasites have definite and varying effects on the shell dimensions, thickness, and secondary shell structures, and because shell development is under the control of specific genes (Marin and Luquet, 2004, Jackson *et al.*, 2006, Jackson *et al.*, 2007), it is potentially affected by epigenetic changes. This topic will be expanded upon later in the general discussion.

### *Aims of the Study*

Given the widespread findings of parasite-induced host manipulation in multiple systems, the current study aims to further investigate the mechanisms behind parasite-induced host manipulation, and whether epigenetics could be one such mechanism. The findings of *W. pipientis* studies show that single-celled parasites are able to exert directed epigenetic modifications in their multi-cellular host, so the next step to build on this knowledge is to investigate whether multi-cellular parasites can provoke a similar epigenetic effect in multi-cellular hosts. Though this study does not aim to induce any causative effects of parasite-induced epigenetic changes in the study system, the main aim is to detect differences in epigenetic patterning of infected host individuals compared to their uninfected conspecifics.

Specifically, I aim to:

- Determine whether multicellular parasitic infection is associated with changes in epigenetic modifications in the host.
- Determine whether epigenetic changes occur in first intermediate gastropod hosts of a trematode; thus perhaps shedding light on the mechanisms behind the change in shell shape observed in infected snails.

It is hypothesised that parasitised hosts will display different epigenetic modifications than their uninfected conspecifics, with various parasite species altering the epigenome (genome plus epigenetic modifications) of their host in distinct ways.

Generally, the present study aims to determine whether epigenetic manipulation is induced by parasitic infection to the benefit of the parasite. Finding such a link would provide valuable information on how parasite-induced host manipulation occurs, how it evolves, and shed light on the genetic interactions between a host and a parasite. Differences in epigenetic modifications between infected and uninfected snails will be investigated at both the DNA and histone level using enzyme-linked immunosorbent assay (ELISA) to determine global DNA methylation levels and mass spectrometry (MS) to determine the patterns of histone modification in infected versus uninfected individuals.

## **2 General Methods**

### *Animal Collection*

*Zeacumantus subcarinatus* snails were collected from Lower Portobello Bay, Otago Harbour, South Island, New Zealand (45° 47'S, 170° 42'E) by hand between May 2010 and May 2011. Animals were collected at low tide times during the day, and were found under rocks and in patches of macrophytes/seaweed beds. Animals were kept alive in the laboratory under natural light conditions in aerated sea water and provided macrophyte material from the sample site to graze on. A total of nine snails, three of each infection status, were used in the study.

### *Tissue Harvesting*

Snails were selected based on shell size, here using shell length as an indication of snail age. Snails of a similar size were chosen, as epigenetic modifications are known to accumulate over an organism's lifetime in other organisms (Fraga *et al.*, 2005). Snails of a similar age are more likely to have a similar amount of epigenetic modifications independently of parasite infection, hence limiting epigenetic modifications accumulated over a normal lifetime as a confounding factor.

Uninfected and *Philophthalmus* sp. infected individuals are rare in the snail population sampled, thus all individuals exhibiting either of those infection statuses were included in the study. *Maritrema novaezealandensis* infected snails were the most common in the sample site, so intermediate-sized snails were selected. Shell length was measured using digital callipers from the shell's apex to the lower edge of the aperture. To determine the infection status of the snails (uninfected, or infected with a particular parasite), the selected snails were individually separated into 6 ml wells containing sea water and incubated under constant light at a temperature of 25°C for at least 8 hours. These temperature and light conditions induce the emergence of mature parasites from the snail host (Kuntz, 1947, Lo and Lee, 1996, Poulin, 2006).

Snail tissue was then harvested by gently crushing the shells open with a hammer, while the snails were placed between two sheets of paper towel. The snail tissue was carefully uncoiled from the shell to extract as much of it intact as possible, as this helps ensure the haemolymph is not lost. The shell fragments were removed and infection status was confirmed. If the snail was found to be infected, the gonads, which are usually the area of highest infection (Martorelli *et al.*, 2008), were completely removed and any stray parasites were removed from the host tissue as best as possible. If the snail was uninfected, the entire

snail was used; however, some snails were also halved, into gonad and somatic tissue sections that were analysed separately. All the snail tissue samples were rinsed twice in distilled water to remove any remaining shell and any attached parasites, and stored in a -80°C freezer ready for further analysis. Contamination between samples was further reduced by thoroughly rinsing the equipment used (petri dishes, forceps, etc.) with distilled water after processing each sample, using clean microarray tubes and autoclaving pipette tips where needed.

### **3 DNA Methylation Analysis**

### 3.1 Introduction

DNA methylation of cytosine bases occurs in many extant lineages (reviewed in Bird, 2002) and occurs when small methyl groups ( $\text{CH}_3$ ) attach to cytosine nucleotide bases on DNA (Jablonka and Lamb, 2005), preferentially in areas of relatively high repeating CpG doublets (cytosine next to guanine with phosphate groups in the middle); these areas are commonly referred to as CpG islands (Gardiner-Garden and Frommer, 1987). CpG islands are often positioned at the 5- ends of genes in humans, appearing as clusters within the normally CpG-depleted bulk DNA (Gardiner-Garden and Frommer, 1987, Adams, 1996, Bird, 2002). Due to the scarce nature of CpG dinucleotides in the DNA of higher eukaryotes, only about 3-4% of total cytosine bases are methylated in mammalian DNA (Iguchi-Arigo and Schaffner, 1989). The covalent addition of the methyl group does not change the function of the cytosine in the genetic code; the protein produced will have the same amino acid sequence despite any modifications on the DNA (Jablonka and Lamb, 2005). Rather, these modifications affect the likelihood that the associated gene sequences will be expressed (Jablonka and Lamb, 2005). In vertebrates, genes in regions of dense methylation, or densely methylated chromatin, are often silenced, i.e. not transcriptionally expressed (Buschhausen *et al.*, 1987, Iguchi-Arigo and Schaffner, 1989, Jablonka and Lamb, 2005). It is hypothesised that DNA methylation in vertebrates induces transcriptional silencing directly by interfering with/preventing transcription factors binding to the promoter, thereby abolishing transcriptional activity (Iguchi-Arigo and Schaffner, 1989, Adams, 1996, Jablonka and Lamb, 2005); or indirectly, by inducing the binding of proteins to methylated DNA which have repressive effects on transcriptional activity and often induce chromatin sequestering (Bestor, 1998, Bird, 2002, Jablonka and Lamb, 2005). For example, four proteins—MBD1, MBD2, MBD3, and MeCP2—belonging to one family have been characterised as having a methylation-dependent repressive effect on transcription (reviewed in Bird, 2002), and also increasing chromatin condensation by further recruiting other proteins which remodel the chromatin structure, thus making the associated DNA sequences less likely to be transcribed (Fuks *et al.*, 2003). Though the intricate mechanisms of DNA methylation have yet to be fully ascertained, the general theme is now becoming increasingly clearer and it is certain that this epigenetic mark is essential in regulating gene expression.

In the animal kingdom, vertebrates have the highest levels of global DNA methylation, i.e. genome-wide DNA methylation; the salmon, frog, chicken, and mouse genomes have 9.1%, 7.2%, 4.5%, and 3.8% of their total cytosine methylated, respectively (Adams, 1996). Conversely, invertebrates have comparatively little DNA methylation

arranged in a stable mosaic of unmethylated and methylated domains (Simmen *et al.*, 1999): the locust has 0.95% total cytosine methylated and the nematode *Caenorhabditis elegans* displays no detectable DNA methylation, also lacking genes which encode the methylating enzyme DNA methyltransferase (Adams, 1996). In single-celled organisms, strain C *Eschericia coli* has 1% total cytosine methylated and 2.1% total adenine methylated (Adams, 1996). Moreover, most bacteria species have methylated adenine as opposed to the methylated cytosine in higher eukaryotes, and a few species have a different form of methylcytosine (Adams, 1996). Conversely, in plants, wheat has 31% total cytosine methylated, though the comparatively high levels of methylation in higher plants may be due in part to the comparatively high abundance of potential cytosine methylation sites (Adams, 1996). Thus, the current scientific knowledge indicates that epigenetic DNA modification is widespread across many living organisms, but specific cytosine methylation is an ancestral trait of eukaryotes and has been lost in many lineages.

It is thought that genome-wide DNA methylation is highly prevalent due its important role as a control mechanism for gene expression (Iguchi-Arigo and Schaffner, 1989). Organised changes in gene expression are brought about by specific methylation patterns, and the maintenance of these changes is often crucial for normal development (Jablonka and Lamb, 2005). The loss of normal methylation patterns has been known to trigger aberrant apoptosis in embryonic mice and *Xenopus* frogs, leading to developmental abnormalities and eventual death (Stancheva *et al.*, 2001, Jackson-Grusby *et al.*, 2001). Moreover, DNA methylation regulates X-chromosome inactivation (Panning and Jaenisch, 1996) and genomic imprinting in many animals (Monk, 1988). As an extension of its role as a transcription regulatory mechanism in vertebrates, DNA methylation has also been proposed as a genome defence mechanism which inhibits the expression of transposon- and retrovirus-encoded genes, and any of the effects on host DNA that these foreign genes can have (reviewed in Mandrioli, 2004). Indeed, when foreign sequences become integrated into the host cell's DNA they are usually silenced and are highly methylated (Adams, 1996, Bird, 2002). Furthermore, there is also evidence suggesting that DNA methylation acts to ensure already silenced genes are permanently silenced (reviewed in Bird, 2002). This gene-silencing role of DNA methylation appears to have been reversed in invertebrates: foreign sequences are unmethylated while the active genes are predominantly methylated (Simmen *et al.*, 1999). Though the specific action of DNA methylation on gene expression varies between vertebrates and invertebrates, this epigenetic modification has an essential role in genomic regulation in both lineages, but the vital role of DNA methylation in development is currently only identified in mammals.

## *DNA Methylation Establishment*

DNA methylation is initiated and maintained by enzymes called methyltransferases which are grouped into distinct families depending on their function and substrate preference/target site specificities (reviewed in Laird and Jaenisch, 1996, Bird, 2002). DNA methyltransferases (DNMTs) act by transferring a methyl group from the donor compound S-adenosyl methionine (SAM/AdoMet) to the target cytosine (Adams, 1996). The cysteine amino acid at the active site of the methyltransferase transfers a methyl group from AdoMet by covalently interacting with the 6<sup>th</sup> carbon atom on the target cytosine, thereby activating the neighbouring 5<sup>th</sup> carbon atom to accept the methyl group (reviewed in Adams, 1996). Three types of active DNA methyltransferases have been characterised in mammals: DNMT1, DNMT3a and DNMT3b. Methylation patterns persist through cell replication cycles, i.e. are heritable, as they are part of the hereditary system; the patterns are replicated as part of the semiconservative replication of DNA by maintenance methyltransferase DNMT1 (Adams, 1996, Jablonka and Lamb, 2005). Specifically, DNMT1 preferentially binds to hemimethylated DNA, copying the parental-strand methylation pattern by attaching a methyl group to the corresponding cytosine of the progeny DNA strand, thus maintaining the original methylation pattern through cell generations. *De novo* methylation, where methyl groups are added to a sequence without a pre-existing methylation, is mainly established by methyltransferases from the DNMT3a and DNMT3b families. Interestingly, DNMT3a has been found to methylate CpA as well as CpG dinucleotides (Ramsahoye *et al.*, 2000). Although the methylation specificity of DNMT3b has not been tested, its high sequence homology with DNMT3a suggests that it too may methylate at non-CpG as well as CpG dinucleotides. In summary, DNMT1 ensures the inheritance of epigenetic marks, and DNMT3a and DNMT3b establish new methylation patterns in response to environmental stimuli.

As mentioned in Chapter 1 epigenetic marks tend only to be stable for a limited time, they are not a permanent fixture of the genome once established; reasons for this are presently unclear. However, early mammalian development periods occur involving genome-wide methylation pattern reprogramming, with a typical large demethylation event followed by periods of cell- and/or tissue-specific patterns of remethylation (reviewed in Reik *et al.*, 2001). These changes are likely due to a combination of active and passive methylation. Typically, a major transformation happens to the paternal genome in the egg cytoplasm with primordial germ cells undergoing the de-methylation/re-methylation event prior to the commencement of DNA replication. This drastic overhaul of primordial germ cell DNA

methylation patterns is necessary as the genomes of mature gametes are highly-methylated compared to somatic cells, and the re-programming event establishes normal genetic imprinting. This reprogramming event is also likely essential for removing parentally-inherited epigenetic modifications and may be necessary for totipotency of the developing embryo cells. In the case of DNA methylation marks outside of reprogramming events, demethylation is hypothesised to occur passively through errors in epigenetic replication as a result of low fidelity for the process (Pollack *et al.*, 1980, Wigler *et al.*, 1981, Pfeifer *et al.*, 1990). This is thought to have a diluting effect on methylation over many cell generations, due to DNA replication without the accompanying replication of methylation patterns. However, an agent directly related to demethylation has recently been discovered: the TET1 protein belonging to a family of ten-eleven translocation (TET) hydroxylase proteins, is known to catalyse the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Tahiliani *et al.*, 2009). Though the mechanism(s) behind the conversion is yet to be defined, it is hypothesised that the addition of the hydroxyl group interferes with DNMT1 and results in a subsequent dilution of methylation; this can be interpreted as TET1 serving an important role in regulating DNA methylation fidelity (Williams *et al.*, 2011). The conversion from 5-mC to 5-hmC may also disassociate or affect the binding of effector proteins (Williams *et al.*, 2011). Moreover, the TET1 protein itself possibly affects DNA methylation directly by binding to regions of high CpG density, thereby physically limiting access of DNMTs (Xu *et al.*, 2011). TET1 also possibly has a role in the removal of aberrant DNA methylations and has is essential for coordinating the expression of genes during development (Williams *et al.*, 2011). The recent discovery of TET proteins and their possible roles in DNA demethylation has filled a long-standing gap in knowledge, as it had been previously thought that active demethylation was unlikely due to the resistant nature of the carbon-carbon covalent bond between the cytosine nucleotide and the methyl group, as well as the apparent lack of agent involved in demethylation (reviewed in Adams, 1996, Bird, 2002).

#### *Examples of Environmental Stimuli inducing changes in DNA Methylation*

As mentioned, *de novo* methylation occurs in reaction to environmental stimuli; though most of the current knowledge of the direct effects of environmental stimuli on DNA methylation is derived from studies in cancer epigenetics. For example, inorganic arsenic can induce cancer development in humans through the intracellular reduction of donor AdoMet levels (reviewed in Sutherland and Costa, 2003). This component is necessary for normal DNA methylation as well as normal metabolism of arsenic, and its depletion in the malignant

tumour cells likely instigates the associated global hypomethylation. In a non-cancer context, much like the classic *Avy* mice discussed in Chapter 1, the maternal environment during pregnancy also has an effect on the activity of the *axin* gene in mice. The *axin* gene is involved with regulation of axial patterning, and, as with *Avy* mice, a promoter-containing mutation upstream of the gene is subject to epigenetic silencing, creating a heritable axin-fused (*Axin<sup>FU</sup>*) allele which results in a kinky tail phenotype when hypomethylated, i.e. active mutant transcription (reviewed in Rakyan *et al.*, 2003 and Waterland *et al.*, 2006). The level of methylation at the mutated promoter is negatively correlated to tail kinkiness, resulting in a range of tail phenotypes, from completely straight (wild-type) to extremely kinked, according to the subsequent expression level of the *Axin<sup>FU</sup>* allele. The intensity of promoter methylation in progeny is responsive to maternal diet during embryogenesis, which is thought to prevent the tail-specific mid-gestation loss of DNA methylation at the mutated promoter, rather than active *de novo* methylation. As is the case with *Avy* mice, this epigenetic trait is inherited vertically through incomplete clearance during embryonic reprogramming and is subject to some genetic imprinting: paternal transmission results in a greater phenotypic expression. This is likely due to the paternal strain being more resistant to reprogramming during embryogenesis. These examples demonstrate that both biotic and abiotic environmental stimuli have the ability to cause direct and measurable epigenetic changes. Such examples demonstrate the effect environmental stimuli can have on the highly reactive DNA methylation system to induce *de novo* methylation, indicating that it is possible parasitic infection can also induce such changes to gene expression.

### *Aims*

This chapter aims to determine whether parasitic infection has any effect on the host's global DNA methylation levels, and whether this may be the mechanism behind the observed parasite-induced host manipulation. The system used here involves two species of trematode in their first intermediate host, i.e. *Philophthalmus* sp. and *Maritrema novaezealandensis* in the marine snail, *Zeacumantus subcarinatus*. Infected hosts in this system exhibit wider width-to-length shell ratios compared to their uninfected counterparts (Hay *et al.*, 2005), which is thought to increase parasite fitness by increasing the shell space available to parasitic proliferation (Lagrue *et al.*, 2007) and thus increasing the likelihood of successful transmission to the next host.

## 3.2 Methods

### *DNA Extraction*

The Qiagen DNeasy® blood and tissue kit was used for the extraction of genomic DNA. Using this kit, I was able to extract DNA from a range of sample sources, including frozen animal tissue and cells, and blood. This makes the kits ideal for the samples used in this study, which were whole, frozen snails with their haemolymph included.

A blunted 1000 µL pipette tip was used to break up the snail tissue into small pieces and allow for lysis. The pipette tip was blunted by heating it and pushing the softened end of the tip into a 1.5mL microcentrifuge tube, thus creating a pestle which fits the shape of the microcentrifuge tube in which the snail tissue was stored. One pestle was created for each sample, to prevent contamination. Each sample was thoroughly ground up in 80 µL of the tissue lysis buffer ATL. After the tissue was broken up sufficiently, a further 280 µL of lysis buffer ATL was added to the sample, along with 40µL of proteinase K, a protein digester and nuclease inhibitor. The sample was vortexed for at least 15 seconds to thoroughly mix the buffer and enzyme through the tissue. The sample was then incubated at 56°C for a minimum of two hours to allow for tissue and membrane lysis. After incubation, the sample was again vortexed for 15 seconds, then 400 µL of buffer AL and 400 µL of 95% ethanol were thoroughly vortexed through the sample for ten seconds. The buffer AL provides salt to neutralize the charge on the DNA, and the ethanol dehydrates the DNA, causing it to precipitate. This increases the affinity of the DNA for the silica membrane of the spin column, aiding in the purification of the sample DNA. Thus, the sample and all of the solution, including any precipitate, was transferred into a DNeasy® mini spin column which was placed inside a 2 mL collection tube, both were included in the kit. This was centrifuged at 6000 g for one minute. The collection tube, along with its contents, was discarded. The column and its contents were transferred into a new 2 mL collection tube. 500 µL of buffer AW1 was added, and this was centrifuged again at 6000 g for one minute. The column and its contents were again transferred into a new 2 mL collection tube. 500 µL of buffer AW2 was added, and this was centrifuged at 20,000 g for three minutes to dry the membrane. Buffers AW1 and AW2 both have a high salt content which increase the affinity of DNA to the column membrane, allowing the DNA to be further isolated. Again, the collection tube, along with its contents, was discarded. The DNeasy mini spin column was then put into a 1.5 mL microcentrifuge tube and 200 µL of elution buffer AE was added directly to the membrane. This was incubated at room temperature for one minute, and then centrifuged for one minute

at 6000 g to elute the sample. The elution buffer is a low-salt solution, causing the DNA to detach from the spin column membrane. The resulting product was stored at 20°C. In all, nine samples were extracted, as each of the three infection statuses: (*Philophthalmus* sp. infected snail (P), *M. novaezealandensis* infected snails (M), and uninfected snails (UN)) consisted of three snails each.

### *DNA Quantification*

The extracted DNA samples were run through a 1% agarose gel to ensure the DNA was confirmed all DNA samples were of equally high quality to be comparable, ensuring degradation did not affect the results. It was also necessary to quantify the amount of double-stranded DNA in each sample, to ensure that equal concentrations of DNA were loaded into each well for the ELISA; an Invitrogen® Quant-iT® dsDNA Broad-Range assay kit was used for quantitation. This assay is highly-selective for double-stranded DNA over RNA, and is designed for assaying samples containing 0.2-100 ng of DNA.

All the assay reagents were allowed to reach room temperature before proceeding, as some of them are refrigerated. A working solution was made by diluting the Quant-iT® dsDNA BR reagent 1:200 in Quant-iT® buffer. With the working solution, two standards were created to calibrate the fluorometer: 10 µL each of standard #1 (0 ng/µL) and standard #2 (100 ng/µL) solutions were added to 190 µL of the working solution. 5 µL of samples UN 1, 2 and 3; P1; and M2 and M3 were added to 195 µL of the working solution. The remaining samples (P2, P3, and M1) required further ethanol precipitation, due to their low DNA yields (see below). All the solutions were vortexed for two to three seconds and then incubated at room temperature for a further two minutes. An Invitrogen® Qubit® fluorometer was used, and was first calibrated with standard #1 and standard #2. The samples were then measured, following the prompts of the fluorometer.

### *Ethanol Precipitation*

Samples P2, P3 and M3 had DNA concentrations too dilute for the size restrictions the ELISA wells imposed, so DNA precipitation was needed. An ethanol precipitation was used in this case: 1/10 volume of 3M sodium acetate ( $\text{NaC}_2\text{H}_3\text{O}_2$ ) was added to the sample. Sodium acetate, a salt, releases sodium ions ( $\text{Na}^+$ ) in solution, which neutralise the positively charged DNA, causing the normally polar and hydrophilic DNA molecules to become non-polar and hydrophobic, hence causing precipitation. The solution was vortexed for two seconds, and

2.5x volume of ice cold ethanol was added. Ethanol in this procedure facilitates the interaction between  $\text{Na}^+$  and the DNA, further helping the DNA to precipitate out of the solution. Due to the low concentrations of DNA in these samples, a longer incubation period was required; hence they were left overnight to precipitate at  $-20^\circ\text{C}$ . The following day, the solution was centrifuged at 20,000 g for 20 minutes to concentrate the precipitate into a pellet. The supernatant was removed, and 500  $\mu\text{L}$  of 70% ethanol was added to remove any residual salts contaminating the DNA sample. The sample was again concentrated into a pellet by centrifuging at 20,000 g for five minutes. The supernatant was removed, and the pellet was left to air dry at room temperature, until all the ethanol had evaporated. The pellet was then re-suspended in elution buffer AL from the Qiagen DNeasy® blood and tissue kit, according to the size of the pellet. Specifically, samples P2 and M3 had similarly sized pellets, and were re-suspended in 30 $\mu\text{L}$  elution buffer; but sample P3 had a smaller pellet, and so was re-suspended in 5 $\mu\text{L}$  of elution buffer AL. The DNA concentration levels according to the fluorometer were used to determine the amount of each sample needed to make a concentration of 200 ng/ $\mu\text{L}$  (when adjusted with ddH<sub>2</sub>O) for each sample for the ELISA.

#### *Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISA is a widely-used assay to detect the presence of an antibody or antigen in a sample, in this case 5-methylcytosine. Generally, a sample is added to a plate of wells, and any antigens present in the sample affix to the well surface; a specific capture antibody is then applied to the wells which bind to the affixed antigens. A detection antibody is then added which binds to the capture antibody; the detection antibody is linked to an enzyme that fluoresces when an enhancer is added.

Here, a MethyFlash<sup>®</sup> Methylated DNA Quantification Kit (Fluorometric) by Epigentek was used. A 1X wash buffer (MF1) was firstly prepared by adding 13 mL of MF1 10x wash buffer to 117 mL of distilled water (pH 7.2-7.5). The MF4 positive control, a methylated polynucleotide containing 50% 5-methylcytosine, (concentration: 20  $\mu\text{g}/\text{mL}$ ) was then diluted with 1x TE buffer (pH 7.5 to 8.0) to 5 ng/ $\mu\text{L}$  (1  $\mu\text{L}$  of MF4 and 5  $\mu\text{L}$  of 1x TE).

The DNA samples were then bound to the wells of the ELISA plate. 80  $\mu\text{L}$  of MF2 binding solution was added to each well, which affixes the DNA in the solution to the well surface. Then, 1  $\mu\text{L}$  of the MF3 negative control, an unmethylated polynucleotide containing 50% cytosine, (concentration: 20  $\mu\text{g}/\text{mL}$ ) and 1  $\mu\text{L}$  of the diluted MF4 positive control were added into the control wells, and 8  $\mu\text{L}$  of the DNA samples were added to the appropriate sample wells. The MF3 and MF4 are controls to which the resulting sample fluorescence is

compared. The solutions in the wells were gently agitated, to ensure the wells were evenly coated with the solutions. The wells were covered with a plate seal and aluminium foil to shield from light, and incubated at 37°C for 90 minutes. After this time, the solution was removed from the wells and each well was washed with 150 µL of the diluted MF1 1x wash buffer each time for three times. This aided in washing off the unbound DNA, so it would not interfere with the result; the wash solution was removed as much as possible.

A capture antibody was then added to identify any 5-methylcytosine (methylated DNA) in the sample. The MF5 capture antibody (concentration: 1000 µg/mL) was diluted at 1:1000 ratio with the previously diluted MF1. 50 µL of this diluted MF5 was added to each well, and the entire well plate was again covered with the plate seal and aluminium foil. This was left to incubate at room temperature for 60 minutes. After the incubation time, the diluted MF5 solution was removed from each well, and the wells were washed with 150 µL of the diluted MF1 1x wash buffer each time for three times to remove any unbound capture antibodies. As much of the wash buffer was removed as possible before the next step.

A detection antibody was then added to bind to the capture antibodies that were, in turn, bound to the methylated DNA. The MF6 detection antibody (concentration: 400 µg/mL) was diluted at 1:2000 ratio with the diluted MF1. 50 µL of this diluted MF6 was added to each well, the well plate was again covered with the plate seal and aluminium foil. This was left to incubate at room temperature for 30 minutes. After incubation, the diluted MF6 solution was removed from each well, and the wells were washed with 150 µL MF1 1x wash buffer each time for four times to remove any unbound detection antibody. As much wash buffer was removed as possible.

An enhancer solution was added to the wells to increase the resulting fluorescence of the sample, by increasing the sensitivity of the detection antibody to the fluorescent enzyme. The MF7 enhancer solution was first diluted to 1:5000 in diluted MF1 solution. 50 µL of this diluted MF7 enhancer solution was added to each well, the well plate was again covered with the plate seal and aluminium foil. This was left to incubate at room temperature for 30 minutes, allowing the enzyme to attach to the detection antibodies and converted to a detectable (i.e. fluorescent) form. After incubation, the diluted MF7 solution was removed from each well, and the wells were washed with 150 µL MF1 1x wash buffer each time for five times to remove any unbound enhancer solution. Each well was then washed with 150 µL of 1x PBS (pH 7.2 ó 7.5) once.

The fluorescence was then measured. A fluoro-development solution was prepared by adding 1 µL of the MF8 fluoro developer and 1 µL of the MF9 fluoro enhancer into 500 µL of MF10 fluoro dilutor. 50 µL of this fluoro-developer solution was added to each well and

the well plate was incubated away from light, using the plate seal and aluminum foil once again, at room temperature for four minutes. The relative fluorescence units (RFU) of the samples and controls were measured and read on a fluoro plate reader, Polarstar Optima fluorescence spectrophotometer (BMG LabTech). This was set at an excitation wavelength of 550 nm and emission of 590 nm. The data was then analysed using MARS data analysis software (BMG LabTech).

#### *Calculation of Relative DNA Methylation*

To determine the relative methylation of DNA (5-mC%) in the samples compared to the controls, the following formula provided in the Kit's user guide was used:

$$5\text{-mC}\% = \frac{(\text{sample RFU} - \text{MF3 RFU}) / S}{(\text{MF4 RFU} \div \text{MF3 RFU}) \times 2 / P} \times 100\%$$

**S** is the amount of input sample DNA in ng.

**P** is the amount of input positive control (**MF4**) in ng.

**2** is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

#### *Statistical Analyses*

A one-way analysis of variance was performed to determine the difference in percentage 5-mC between *Z. subcarinatus* individuals of different infection status (uninfected, infected with *Philophthalmus* sp. and infected with *M. novaezealandensis*). The data was converted using the above formula was used for the comparison. Minitab version 15 was used to perform the analysis.

### 3.3 Results

There were no significant differences in mean 5-mC levels of the uninfected *Z. subcarinatus* snails (U) compared to both *Philophthalmus* sp.-infected snails (P) and *M. novaezealandensis* -infected snails (M) (1 factor ANOVA,  $F_{2,6} = 0.11$ ,  $p = 0.895$ ; fig. 3). However, there was a lot of variation within and between the infection status groups. The mean 5-mC levels of each infection status were: 0.52% for uninfected snails, 0.62% for *Philophthalmus* sp.-infected snails, and 0.58% for *M. novaezealandensis* -infected snails. The overall mean 5-mC level of all samples was 0.57%, and none of the individual samples had a mean 5-mC level surpassing 1.0% (fig. 3).

The mean shell size for uninfected *Z. subcarinatus* snails was  $154.3 \text{ mm} \pm 2.4$  (mean  $\pm$  standard error;  $n = 20$ ), with snails infected with *M. novaezealandensis*  $153.9 \text{ mm} \pm 1.6$  (mean  $\pm$  standard error;  $n = 20$ ) and snails infected with *Philophthalmus* sp.  $167.6 \pm 4.26$  (mean  $\pm$  standard error;  $n = 7$ ). A subset of these snails was used in the experiments.

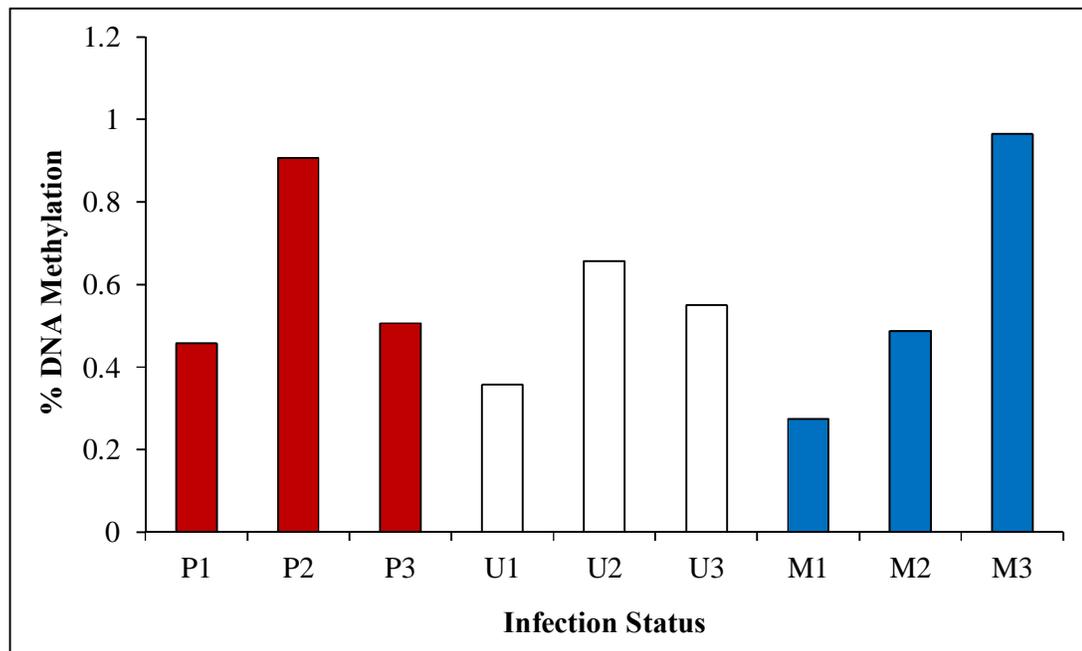


Figure 3. Percentage overall DNA methylation for whole *Zeacumantus subcarinatus* individual snails which are uninfected (Ux), infected with *Philophthalmus* sp. (Px), and infected with *Maritrema novaezealandensis* (Mx).

### 3.4 Discussion

In the current study, no significant differences in overall genomic 5-mC levels were detected between uninfected *Zeacumantus subcarinatus* snails and snails infected with either *Philophthalmus* sp. or *Maritrema novaezealandensis*. The mean overall methyl-cytosine found here of <1% is consistent with the existing data on invertebrate genome 5-mC levels which, as previously mentioned, are often less than 1% of the genome (Adams, 1996). The findings of the current study indicate that either (1) the parasitic infection is having no effect on its host's DNA methylation level, or (2) parasitic infection is causing targeted changes at specific genes and therefore not causing a detectable DNA methylation global change. These specific changes in DNA methylation patterning possibly induce the observed phenotypic changes in the host.

#### *DNA methylation patterning in Cancer Epigenetics*

The essential regulatory roles of DNA methylation in gene expression during processes such as development mean that any mutations affecting normal methylation patterns have a huge potential to lead to serious disease, and is indeed central to many human diseases including cancer (reviewed in Liu *et al.*, 2010). Almost all human cancer tumours undergo an overall genome-wide loss of DNA methylation, a several-fold increase in DNMT1 levels, and a shift in methylation pattern due to aberrant hypomethylation of cell growth genes and tumour-suppressor genes and hypermethylation of genes which control apoptosis (reviewed in Adams, 1996, Issa, 1999, Brown and Stratthdee, 2002, Esteller, 2007). Though the affected genes are not always directly linked to tumour progression, the changes in gene function are thought to provide a permissive environment aiding tumour progression (reviewed in Baylin *et al.*, 2001). Interestingly, each tumour subtype appears to have an almost distinctive pattern of CpG-island hypermethylation, making up a unique DNA hypermethyome distinct to the individual malignancy (reviewed in Brown and Stratthdee, 2002, Esteller, 2007). Methylation itself can also be the cause of coding mutations, as it is capable of undergoing spontaneous C → T transitions resulting from hydrolytic deamination (reviewed in Jones and Baylin, 2002). Parallels can be drawn between disease and parasitism, as they have similar negative effects on the organism affected; it is possible, then, that they may also have similar effects on the genome, given the high reactivity of the DNA methylation system. Hence, it appears to be more important to address the pattern of DNA methylation marks when investigating differences in this system between samples, though the current study has addressed an

important issue by establishing that a multicellular parasitic infection does not induce global changes in host DNA methylation levels.

### *DNA Methylation in Invertebrates*

Given that the importance of DNA methylation patterning has largely only been focused on vertebrate systems, and considering the differences in the role of invertebrate DNA methylation, it could be argued that applying the same experimental theories to invertebrate systems is naive. As mentioned in Chapter 1, DNA methylation in invertebrates has significant differences to DNA methylation in vertebrates, the major differences being the mosaic pattern of DNA methylation in invertebrates, and its role in as a gene activator (Simmen *et al.*, 1999, Mandrioli, 2007). However, though reversed, invertebrate DNA methylation has a similarly important gene-activating function which is speculated to focus the site of transcription and reduce the rate of aberrant of transcription, known as transcriptional interference (reviewed in Simmen *et al.*, 1999). This reversed role of DNA methylation in invertebrates may be connected to the difference in the DNA methylation target: methylation in vertebrates occurs at the promoters, whereas methylation in invertebrates occurs on the cytosines within the coding section of the genes (reviewed in Mandrioli, 2007). In the same manner, invertebrates do not use DNA methylation as a transposon defence mechanism to silence foreign repetitive DNA sequences as in vertebrates (reviewed in Simmen *et al.*, 1999). Methylation in invertebrates is also not limited to CpG sites, as CpA-, CpT- and CpG doublets have also been found to be targets of methylation (reviewed in Mandrioli, 2004).

It is thought that the differences in DNA methylation function between vertebrates and invertebrates are due to invertebrates retaining the ancestral role of DNA methylation of preventing aberrant gene transcription (reviewed in Mandrioli, 2007). Despite the differences in the role of DNA methylation between vertebrates and invertebrates, the studies mentioned above suggests that DNA methylation in invertebrates plays a similarly essential role in genome regulation. Thus, assessing the pattern of genome DNA methylation is likely a relevant and informative approach to take when comparing the differences between invertebrate treatment samples. Given that DNA methylation in invertebrates and vertebrates likely has a common ancestor, changes in the patterning of DNA methylation in invertebrates would likely have similarly negative effects as it does in vertebrates.

## *Conclusions*

In light of the literature in cancer epigenetics, it would seem that the global level of DNA methylation is not a decisive factor in epigenetic regulation, rather, the various methylation marks form patterns of active and silenced genes that determine the overall phenotypic outcome. Therefore, while no significant global differences were found in the current study between infected and uninfected snail hosts, this does not necessarily mean the trematode parasites are not having any effects on host DNA methylation; indeed, there could be changes in methylation patterning not detectable given the host, *Z. subcarinatus*, has yet to have its genome sequenced. Most of the current knowledge of DNA methylation is based on studies on vertebrates, and invertebrates are known to use DNA methylation in strikingly different, and sometimes opposing, ways. However, given that invertebrates likely retained an ancestral form of DNA methylation once shared by vertebrates, it is plausible that DNA methylation patterning has a similar essential role in phenotypic outcome.

## **4 Analysis of Histone Post-translational Modifications**

## 4.1 Introduction

As with DNA methylation, post-translational modifications (PTMs) on histones also occur in reaction to environmental stimuli, and are significant transcriptional regulators (Rice and Allis, 2001). Due to the role of histone PTMs in chromatin packaging, hence gene expression, and also the reactivity of this epigenetic system to environmental stimuli, parasites could possibly induce phenotypic changes in their hosts by causing changes in chromatin structure. Chromatin is made up of nucleosomes which are, in turn, made up of 147 base pairs (bp) of DNA wrapped almost twice around a histone core octamer; these core histones are some of the most evolutionarily conserved eukaryotic proteins (Wolffe and Pruss, 1996). The octamer is comprised of the carboxyl-terminal globular domains of the core histones in a (H3-H4)<sub>2</sub> tetramer formation flanked by two H2A-H2B dimers (Peterson and Laniel, 2004, Villar-Garea and Imhof, 2006, Campos and Reinberg, 2009); and each nucleosome is separated by approximately 10-60 base pairs of linker DNA (Elgin and Grewal, 2003). Each histone also has a highly basic tail comprised of a 20-35 amino acid terminal (N-terminal) extending from the surface of the nucleosome core (Rice and Allis, 2001, Villar-Garea and Imhof, 2006, Campos and Reinberg, 2009). These histone tails are responsible for binding DNA to the histone structure and also have a role in histone-histone, hence inter-nucleosomal, interactions (reviewed in Peterson and Laniel, 2004). Although the tails do not make a large contribution to the structure or stability of the nucleosome, they are essential for the complex folding of the nucleosomal arrays (reviewed in Peterson and Laniel, 2004). However, this tight folding poses a major obstacle to gene transcription as it blocks access of the necessary transcriptional machinery (Rice and Allis, 2001). The lysine and arginine rich structure of the histone tails helps overcome this restriction, as these amino acids are subject to a high degree of PTM which regulates gene expression by inducing changes to chromatin structure. PTMs occur through the covalent bonding of chemical groups mainly to the amino-terminal histone tails, with a few bonding to the central histone globular domains (Peterson and Laniel, 2004, Berger, 2007, Campos and Reinberg, 2009, Smith and Denu, 2009). Histone tails are subject to many types of PTMs including phosphorylation, ubiquitylation, ribosylation, and SUMOylation of various amino acids, and methylation and acetylation of arginine and lysine amino acids (Peterson and Laniel, 2004, Berger, 2007); the latter two are the most well studied and will be the focus of the current study. Generally, histone acetylation is associated with transcriptional activation whereas methylation can either activate or suppress transcription depending on the target site (reviewed in Berger, 2002, Stewart *et al.*, 2005). These histone PTMs are thought to directly influence dynamic changes in chromatin structure

by affecting histone interactions with DNA and chromatin-associated effector proteins (reviewed in Rice and Allis, 2001). However, it is still uncertain how these histone PTMs functionally alter nucleosomal structure and exactly how they affect chromatin folding (reviewed in Peterson and Laniel, 2004). But it is certainly likely that these modifications have a significant biological effect, given that the precise organisation of chromatin has a large impact on cellular processes through the regulation of gene transcription reviewed in (Peterson and Laniel, 2004). Indeed, these PTMs can affect many biological function such as: chromosome segregation, DNA replication and recombination, and the detection of DNA damage (reviewed in Rice and Allis, 2001, Bannister and Kouzarides, 2005; see table 1).

Table 1. Examples of histone modifications and their likely function. This list is non-exhaustive and limited to cases identified in animals. K denotes lysine and R denotes arginine amino acids, enzymes which bind/remove the histone post-translational modifications are also included. Adapted from (Peterson and Laniel, 2004).

Modification	Histone	Site (animals found in)	Enzyme	Possible function	
Acetylation	H2A	K5 (mammals)	Tip60, p300/CBP	Activation	
		H2B	K5	ATF2	Activation
	K12 (mammals)		p300/CBP	Activation	
	K15 (mammals)		ATF2	Activation	
	H3	K20	p300	Activation	
		K4	Esa1	Activation	
			Gcn5	Activation	
		K9	SRC-1	Activation	
			K14	Gcn5, PCAF	Activation
				Esa1, Tip60	Activation, DNA repair
		K18	SRC-1	Elp3	Transcription elongation
				hTFIIIC90	RNA polymerase III transcription
	TAF1		RNA polymerase II transcription		
	p300		Activation		
	Gcn5		Activation, DNA repair		
K23	K18	(SAGA/STAGA complex)	DNA replication, Activation		
		p300, CBP	Activation		
	K27	Gcn5	DNA repair		
		Sas3	Activation/elongation		
H4	K5	p300, CBP	Activation		
		Gcn5	Histone deposition		
	K5	Hat1	Activation, DNA repair		
			Esa1, Tip60	Activation	
			ATF2	Activation	

				p300	Activation
			K8	Gcn5, PCAF	Activation
				Esa1, Tip60	Activation, DNA repair
				ATF2	Activation
				Elp3	Transcription elongation
				p300	Activation
			K12	Hat1	Histone deposition, Telomeric silencing
				Esa1, Tip60	Activation, DNA repair
			K16	Gcn5	Activation
				Tip60	DNA repair
				ATF2	Activation
				Sas2	Euchromatin
Methylation	H3	R2 ( <i>Homo sapiens</i> )	K4	PAD14	demethylase/deiminase
				Set9	Active euchromatin, Transcriptional elongation/memory, Activation
				( <i>Homo sapiens</i> )	
				LSD1	Transcriptional repression, demethylase/deiminase
				MLL, Trx	Activation
		R8 ( <i>Homo sapiens</i> )	K9	PAD14	demethylase/deiminase
				Suv39h, Ctr4	Silencing, DNA methylation
				G9a	Transcriptional repression, Imprinting
				SETDB1	Transcriptional repression
				Dim-5, Kryptonite	DNA methylation
				( <i>Homo sapiens</i> )	
				LSD1	Activation, demethylase/deiminase
			R17 ( <i>Homo sapiens</i> )	PAD14	demethylase/deiminase
			R26 ( <i>Homo sapiens</i> )	PAD14	demethylase/deiminase
			R17	CARM1	Activation
			K27	Ezh2	Activation, X inactivation
			K36	Set2	Transcriptional elongation
			K79	Dot1p	Euchromatin
	H4		R3	PRMT1	Activation
				( <i>Homo sapiens</i> )	
				PAD14	demethylase/deiminase
				(mammals)	
				JMJD6	mono/di-demethylase
			K20	PR-Set7	Transcriptional silencing
				Suv4-20h	Heterochromatin

### *Histone Modification mechanisms*

Various hypotheses exist as to the mechanisms by which PTMs either activate or repress transcription. One hypothesis posits that PTMs cause direct structural changes to chromatin by affecting the basicity, hydrophobicity and electric charge of the histone tails, thereby decreasing their affinity for negatively charged DNA (reviewed in Rice and Allis, 2001). But given the relatively small size of methyl groups, they likely do not have a

significant effect on the lysine or arginine residues to which they bind (see Peterson and Laniel, 2004, Bannister and Kouzarides, 2005). Non-coding RNAs are also thought to have a role in propagating changes in chromatin structure, having the ability to elicit gene expression and silencing (reviewed in Elgin and Grewal, 2003). The current leading hypothesis postulates that histone PTMs cause a more indirect functional change through mediating associations between amino-terminal tails and histone binding effector proteins, which activate chromatin remodelling (reviewed in Wolffe and Pruss, 1996, Berger, 2002, Berger, 2007). It is thought that the PTMs on histone amino-tails alter nucleosome surfaces, thereby creating binding sites for specific protein-protein interactions (e.g. acetyl-lysine-binding bromodomains) and regulating the access of proteins to the chromatin fibre to stimulate repression or activation (reviewed in Berger, 2002, Peterson and Laniel, 2004). Explicitly, the transcriptional state of chromatin is determined by the specific PTMs carried on the various amino acids on the histone tails. These specific modifications have correspondingly specific enzymes responsible for attaching (methyl- or acetyl-transferases; see table 1) or removing them (demethyl or deacetylases; see table 1 and Smith and Denu, 2009 for a review); these enzymes bind to PTMs via a set of highly conserved chromatin domains: chromodomains bind methylated histones and are associated with gene silencing, whereas bromodomains are more specialised, binding acetylated lysines and are generally associated with gene transcription (reviewed in Berger, 2002, Quina *et al.*, 2006); arginine does not undergo acetylation (reviewed in Smith and Denu, 2009). These chromatin domains induce the further binding of chromatin-remodelling proteins which have either silencing (e.g. heterochromatin protein 1 (HP1) (Stewart *et al.*, 2005) and polycomb complex group (PcG) (Ringrose and Paro, 2004) or activating properties (e.g. trithorax-group proteins (TRX) (Ringrose and Paro, 2004); see Figure 4 for diagram illustrating this process. To more clearly illustrate this hypothesis, the process of gene activation by the co-activating heterochromatin protein 1 (HP1) will be described.

The HP1 protein is one of the most well-researched chromatin proteins. HP1 specifically binds to methylated lysine 9 on histone H3 (H3K9) via its highly-specific amino-terminal chromodomain (reviewed in Elgin and Grewal, 2003, Berger, 2007) and recruits the lysine 9-specific histone methyltransferase of the Su(var)3-9 family through its carboxy-terminal chromo shadow domain to further methylate the amino acid (reviewed in Berger, 2002, Elgin and Grewal, 2003, Richards, 2006). Though HP1 is the best characterised heterochromatin-associated non histone protein in eukaryotes, the exact mechanism by which it induces and propagates the heterochromic state remains unclear. Multiple lines of research

reveal HP1 has associations with various systems, by: promoting silencing by association with membrane receptors on the nuclear envelope, associating with other heterochromatin proteins, acting to anchor or link chromatin subunits, or condense chromatin structure by self-aggregating through the chromo shadow domains (reviewed in Yamamoto and Sonoda, 2003, Eissenberg and Elgin, 2000). This lack of knowledge of one of the most well-studied chromatin proteins reflects the current knowledge of histone PTMs: the major themes have been discovered, but the exact mechanisms by which they work and where they fit in with the other identified associated molecules yet to be elucidated.

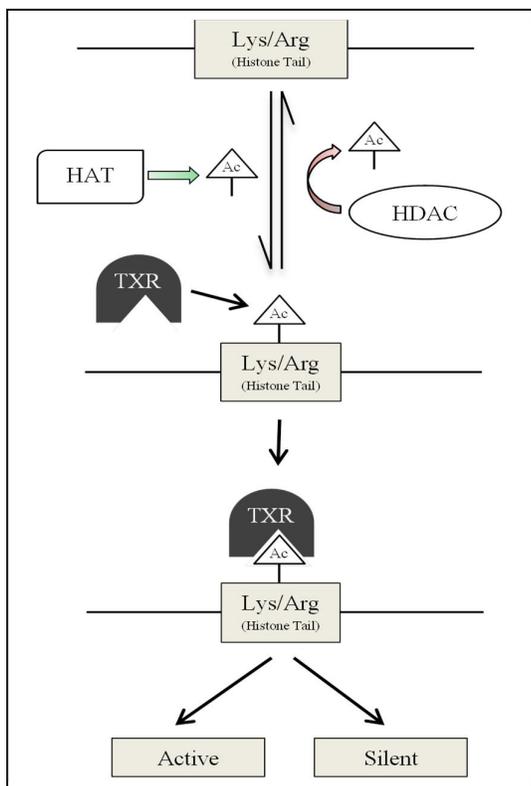


Figure 4. How histone acetylation and deacetylation affect chromatin structure. Lysine or arginine amino acids in the histone tails can be acetylated by a histone acetyltransferase (HAT) and can be removed by an antagonistic histone deacetylase (HDAC). Following acetylation, the acetyl modification recruits and binds to a conserved chromatin domain within a transcriptional regulator protein (TXR), also known as an effector protein. The effector protein recruited by the specific histone post-translational modification determines whether transcription is active or silent. Adapted from Rice and Allis (2001)

### *Examples of Environmental Stimuli inducing Histone Epigenetic changes*

As mentioned, environmental abiotic and biotic stimuli can induce phenotypic changes by affecting histone PTMs. The potent animal carcinogen nickel is thought to exert an epigenetic effect by altering gene expression via heritable changes in DNA methylation and histone acetylation. Although its exact mechanism is unknown, *in vitro* studies have shown nickel to bind almost exclusively to histones and non-histone proteins in heterochromatin within the nucleus (reviewed in Sutherland and Costa, 2003). The epigenetic effect of maternal behaviour on rat pups puts this histone modification into a biological perspective. Adult rats that experienced high levels of maternal care, maternal licking and

grooming and arched-back nursing (LG-ABN), during the first week of lactation are generally less fearful and show more modest responses to stress than pups which experienced low maternal care (Liu *et al.*, 1997, Stern, 1997, Caldji *et al.*, 1998, Francis *et al.*, 1999, Weaver *et al.*, 2004). These long-term effects on offspring gene expression are facilitated by changes in both chromatin structure and DNA methylation. Specifically, pups exposed to high LG-ABN rates have significantly lower levels of DNA methylation at the glucocorticoid receptor transcription factor (NGFI-A) binding site, compared to pups exposed to lower LG-ABN (Weaver *et al.*, 2004); thus, glucocorticoid receptor gene expression is higher in the high LG-ABN pups, leading to a lowered stress response compared to their low LG-ABN conspecifics. Furthermore, adult offspring of high LG-ABN mothers also have significantly higher levels of H3K9 acetylation compared to offspring of low LG-ABN mothers; resulting in significantly increased binding affinity of NGFI-A to the glucocorticoid promoter. These epigenetic differences resulting from early-life maternal care exposure are facilitated by targeted DNA methylation at the promoter and histone acetylation which alter chromatin structure. Histone acetylation appears to be an important part of this system, as the addition of a HDAC inhibitor in offspring exposed to low maternal care produced glucocorticoid receptor expression levels comparable to those exposed to high maternal care. Cross fostering confirmed that maternal behaviour, i.e. amount of care given, is the causative factor (Francis *et al.*, 1999).

Conclusively, though the exact mechanism behind how histone PTMs affect gene expression is not yet fully understood, their role in the regulation of gene expression is undeniably significant. The environmentally-induced changes in chromatin structure described have the ability to induce various behavioural and physical phenotypic outcomes and are often linked to diseases such as cancer. Given such empirical results, it is likely that parasites, being significant biotic stimuli with broad physiological impacts, also have a similar effect on histone tail PTMs.

This experiment aims to determine whether parasitic infection has any effect on the host's pattern of global histone modifications and whether this may be the mechanism behind the observed parasite-induced host manipulation. The system used here involves two species of trematode in their first intermediate host, i.e. *Philophthalmus* sp. and *Maritrema novaezealandensis* in the marine snail *Zeacumantus subcarinatus*. Infected hosts in this system exhibit wider width-to-length shell ratios compared to their uninfected counterparts (Hay *et al.*, 2005), which is thought to increase parasite fitness by increasing the shell space available to parasitic proliferation (Lagrué *et al.*, 2007) and thus increasing the likelihood of successful transmission to the next host. A thorough examination of every detectable amino

acid modification is provided and compared between treatment groups (uninfected snails, and snails infected with one of the other parasite). Mass spectrometry and liquid chromatography are used here, as this approach has proven to be very useful for detecting molecules at low levels, as is the case for histone PTMs and is widely used to study differences in amino acid modification profiles.

## 4.2 Methods

### *Shotgun Proteomics for Protein Identification*

Shotgun protein sequencing was used as an untargeted approach to find PTMs in histones. An overall measure of the histone modification profile was acquired, rather than a targeted, more inherently biased approach. Shotgun proteomics involves high-throughput identification of proteins in complex mixtures, using a combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Mass spectrometry is highly accurate (with a mass accuracy in the range 3-10 ppm) and very specific, making it particularly useful in comprehensive protein identification and PTM localisation. Due to the great sensitivity of modern mass spectrometry instrumentation (usually in the low fmol range), low abundance proteins and their PTMs can be identified. Moreover, detection of these modifications can also be achieved with high efficiency (reviewed in Marcotte, 2007). Hence, MS is particularly useful for the present investigation, looking at PTMs in relatively small invertebrates. Three samples from each infection status were used, i.e. three samples each of: uninfected whole *Zeacumantus subcarinatus* snails (U), uninfected somatic tissue (U 0.5), uninfected gonadal tissue (Gon), *Philophthalmus* sp.-infected *Z. subcarinatus* snails (P), and *Maritrema novaezealandensis*-infected *Z. subcarinatus* snails

### *Histone Extraction*

A specific protocol for extracting invertebrate histones is not yet known, so a series of preliminary trials allowed a general Honey bee extraction protocol to be adapted and optimised for snail protein extraction. 500µl Buffer A (see Chapter 6) and 50µl Nonidet P40 substitute was added to the frozen snail tissue to aid in cell lysis, attaining a final NP40 concentration of 0.05%. The tissue was then homogenised with pestle A in a dounce homogenizer, to break open the cells to access the nuclei. The resulting mixture was distributed on top of a 1 ml 1.8M sucrose bed and centrifuged at 20,000 g for 20 minutes at 4°C to purify the nuclei. The supernatant was removed, being diligent in removing all of the waste material on the top of the sucrose, and the pellet was rinsed with 500 ml of Buffer A to rinse off any excess sucrose. 500 µl of 0.4N sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) was used to dissolve the pellet; this was left to incubate at 4°C for a minimum of one hour.

The solution was then spun at 20,000 g for five minutes at 4°C to concentrate the precipitate into a pellet, and the resulting supernatant transferred to a clean microcentrifuge

tube. The supernatant at this point contains the extracted proteins in suspension. 500 µl of 60% TCA (w/v) was added to precipitate the proteins; this was left overnight at 4°C.

The next day, the solution was spun at 20,000 g for ten minutes at 4°C to concentrate the proteins in the precipitate, the supernatant removed, and the resulting pellet was pipetted up and down, then vortexed in 500 µl of acetone, to thoroughly rinse the pellet with the acetone. The solution was centrifuged at 20,000 g for ten minutes at 4°C to re-concentrate the proteins into a pellet, the supernatant was removed, and the pellet was left to air dry off the acetone for 15 minutes. The pellet was then re-dissolved in 50 µl distilled water. The snail tissue was sonicated for 5 minutes and physically fragmented with a blunted 200 µl tip (blunting was achieved by heating an autoclaved 200µl pipette tip and then pressing the softened tip into the bottom of a 200 µl PCR tube) to assist in dissolving the pellet. Although it is acknowledged that some of the proteins would have been lost due to the physical abrasion, it proved an essential step to effectively dissolve the pellet.

Because the amount of tissue was small, the entire organism was used so that an acceptable amount of histones would be extracted. However, the protocol used was not specific enough to extract histones alone, and this resulted in other, undesired, proteins in the resulting sample. Hence, sodium dodecyl sulfate ó polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the various proteins in the sample based on their size.

#### *Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE is widely used to reduce sample complexity in mixtures such as whole-tissue lysates (Yates *et al.*, 2009), which allows for efficient large-scale protein identification. A protein mixture is run through a gel, separating the various proteins in the sample based on their approximate molecular size; thus excluding many undesired proteins and allowing the proteins of interest to be isolated for further analysis (for a review of 2D SDS-PAGE see Herbert *et al.*, 2001).

The SDS component is a dissociating agent used to denature native proteins to individual polypeptides. Because SDS is an anionic detergent that denatures secondary and non-disulfide ó linked tertiary structures, polypeptides, after being treated with SDS, become a rod-like structure which ensures they run uniformly through the gel. SDS also applies a net negative charge per unit length to each protein proportionate to its mass; this negative charge is able to negate any intrinsic charges of the peptide. Therefore, each protein is uniformly tagged to run through the gel, toward the positive electrode, with the distance run dependent on its size. A molecular size marker is usually run alongside the samples, thus a rough

estimation of protein molecular size is given. Due to proteins having varying isoelectric points and molecular weights particular to their primary structure, without SDS treatment, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio. After electrophoresis the resulting gel material can be digested for further analysis. Given these benefits, the SDS-PAGE system can be used with confidence for a wide variety of proteins (Weber and Osborn, 1969).

### *Making the Gel*

A relatively high 15% acrylamide resolving gel was made for separating the proteins, as increased acrylamide concentration results in a smaller pore size in the gel, which is needed to separate small proteins such as histones. The gel is formed by the action of the bisacrylamide in the acrylamide solution which acts to form cross-links between two polyacrylamide molecules.

The stacking gel has a larger pore size (as it only contains a 4% concentration of 30% acrylamide solution) and a lower pH than the resolving gel, this helps the SDS coated proteins in the sample to concentrate seven-fold into a thin starting zone, thus ensuring the resulting, resolved bands are sharper and more defined and the samples start off at the same approximate position so their end positions are therefore comparable. The stacking gel was poured so that it fell 1 cm below the bottom of the wells (Figure 5). Gels were cast no more than 24 hours prior to use, according to the recipes in Chapter 6. To save time, the resolving and stacking gels were made with differing amounts of glycerin; the resolving gel containing more than the stacking gel, so that the stacking gel would float on top of the resolving gel, and thus the two gels could be poured together.

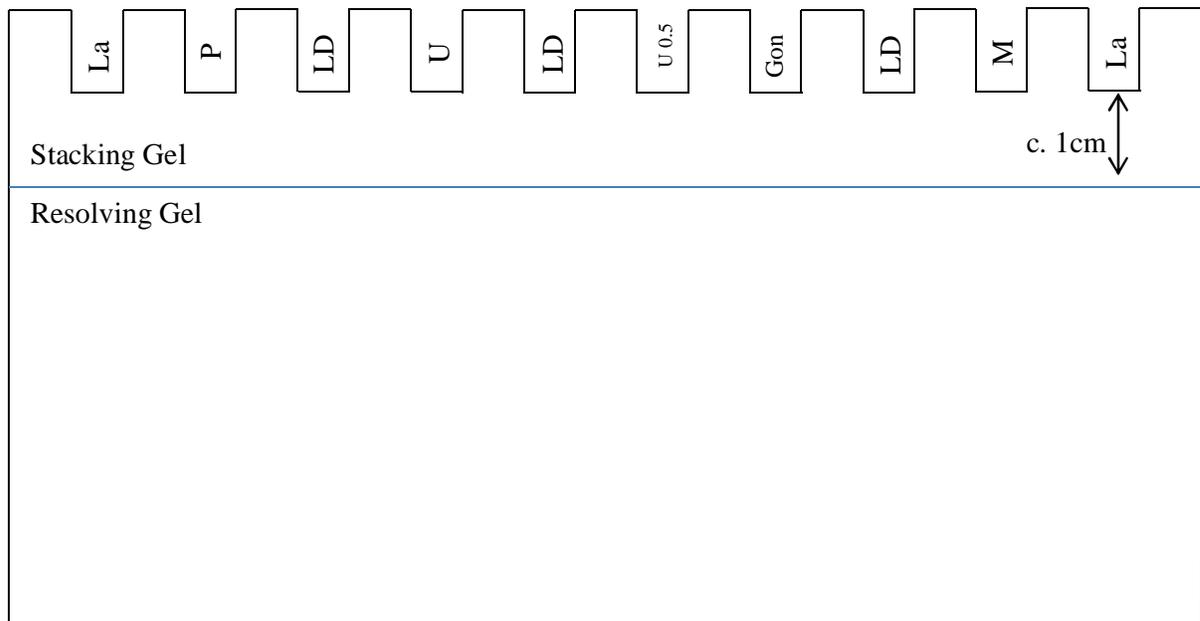


Figure 5. Diagrammatic representation of the gel pouring and sample loading formation for all replicates in the study. The stacking gel (containing 4% of acrylamide (30%) solution) was poured 1 cm below the bottom of the wells. The resolving gel (containing 15% of acrylamide (30%) solution) made up the rest of the gel. Samples included uninfected whole *Zeacumantus subcarinatus* snails (U), uninfected somatic tissue (U 0.5), uninfected gonadal tissue (Gon), *Philophthalmus* sp.-infected *Z. subcarinatus* snails (P), and *Maritrema novaezealandensis*-infected *Z. subcarinatus* snails (M). 7  $\mu$ l of 6x SDS sample buffer (LD) was loaded into empty wells and 7  $\mu$ l of Benchmark® pre-stained ladder (La) was loaded into wells one and ten.

### *Running the Gel*

Each gel served as a single replicate, with each containing all of the snails of varying infection statuses to be compared (see fig. 5). However, because the resulting sample from the histone extraction protocol was a 50  $\mu$ l solution which was too large for a single well in one gel, each the snail sample had to be split into two samples. Therefore, two gels were poured and run simultaneously, each gel containing one complete set of the snail samples of interest. So, each individual gel of the set contained half of each of the samples to be compared (Figure 5).

Thus, each sample was halved into two tubes of 22  $\mu$ l, with 5  $\mu$ l of 6x SDS sample buffer and 3  $\mu$ l of 200 mM Dithiothreitol (DTT) added; the DTT concentration was therefore diluted by approximately 10 fold. DTT is a reducing agent that disrupts disulfide bonds, ensuring the proteins are fully denatured and, combined with the denaturing effect of the SDS, ensuring they run uniformly. The samples were heated at 95°C for five minutes and chilled at 4°C for one minute; this heating helps the SDS bind to the molecules, allowing the detergent

to wrap around the polypeptide backbone. All 30  $\mu$ l of each sample was then loaded into a well, avoiding any un-dissolved pellet, along with 7  $\mu$ l of Benchmark® pre-stained ladder in wells one and ten to help determine when to stop the gel, and 7  $\mu$ l of 6x SDS sample buffer into any empty wells (Figure 5).

10x running buffer (see Chapter 6) was diluted ten-fold and poured into the gel container until the buffer covered the gel cassette, and the gel tank was filled to the pre-determined fill line. The gel was run in a Bio-Rad® protein gel system at 60 volts for 30 minutes through the stacking gel, then at 200 volts until the pre-stained ladder had reached the bottom of the gel on both sides. The gel was then removed from the gel plates and rinsed for five minutes at a time in distilled water three times to remove any residual running buffer, and then stained in colloidal coomassie blue stain (see Chapter 6) for a minimum of two hours. The stained gel was then rinsed with distilled water twice for five minutes at a time with gentle agitation, then agitated in distilled water overnight to destain. Staining of the gel allows visualization of the separated proteins, as the dye molecules in the colloidal coomassie binds to proteins. A colloidal coomassie stain was used as this type of stain only targets proteins, leaving the rest of the polyacrylamide gel unstained; hence, the polyacrylamide gel needed no specific destain, distilled water sufficing as the  $\text{-destain}$ .

Because the proteins were charged according to their size, the smaller the proteins were, the more easily they fit through the pores of the gel, the less resistance encountered. This means that the proteins will have differently migrated through the gel based on their size, and smaller proteins will have travelled farther from their point of origin. The ladder which was run alongside the samples helped determine the weight of the unknown proteins in the sample; this helped identify which bands were likely to contain histones. Histones are smaller than 14kDa, so the bands which migrated beyond that level, according to the ladder, were deemed likely to contain histones and were selected and excised for protease digestion.

### *Tryptic Digestion*

Protease digestion, typically using trypsin, is essential so that each protein can be fragmented to smaller peptides, and hence be more accurately identified. Trypsin acts on the peptide bonds of the carboxyl group of lysine and arginine, cleaving between these two amino acids. Because determining the weight of whole intact proteins is insufficient for accurate identification, proteins are fragmented into peptides, which are more accurately sequenced than whole proteins. As mentioned, the bands which were in the size range of histones were selected; these were the bands which ran beyond band 7 on the protein ladder (i.e. below 25.9

kDa) which was run alongside the samples. These were cut out as close to the edge of the protein band as possible to reduce the amount of acrylamide, as this can interfere with the MS accuracy. These bands were then cut into smaller pieces, approximately 1mm<sup>2</sup> and then subjected to in-gel tryptic digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi, Intavis AG, Cologne, Germany). The protocol for automated in-gel digestion is based on the method of Shevchenko *et al.* (2007). Eluted peptides were dried using a centrifugal concentrator.

NB: the following three procedures, i.e. LC-MS/MS, tandem mass spectrometry and data analysis, were performed by the team at the Centre for Protein Research at the University of Otago.

#### *Ionisation of Tryptic peptides using LC-MS/MS*

The eluted peptides were then further processed using reversed-phase (RP)  $\delta$ HPLC, a common procedure used to separate and purify peptide fragments resulting from the enzymatic digestion of a protein; with separation based on the hydrophobicity of the peptides in the mixture. This additional separation step aids in identification of the protein's amino acids and the PTMs associated with them. The tryptic peptide mixture is pumped, in small volumes, through an RP-HPLC column which contains an immobilized matrix with a hydrophobic surface. The non-polar, and therefore hydrophobic, peptides adhere to this matrix, whereas the polar, hydrophilic peptides remain in the solution/buffer pumping through the column, going on to be ionised in the in-line coupled electrospray unit. Peptide fragments are gradually eluted from the matrix in the column by incrementally increasing the organic solvent concentration, and therefore increasing the hydrophobicity, of the elution buffer, typically using acetonitrile or methanol. The peptide fragments affixed to the hydrophobic matrix elute off when the peptide exhibits a higher affinity for the eluting buffer than the immobilized matrix. This partial separation of the mixture by liquid chromatography (LC) also acts to slow down the flow of analytes to a pace at which effective MS can occur, thus increasing the number of peptides which can be identified by MS/MS (Herbert *et al.*, 2001). Once eluted, these ions also go on to be ionised with the directly-linked electrospray unit (see Yates *et al.*, 2009). Here, samples were re-solubilised in 5% [v/v] formic acid in water and injected onto an Ultimate 3000 nano-flow uHPLC-System (Dionex Co, CA) that was in-line coupled to the nanospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated on an in-house packed emitter-tip column

(75  $\mu\text{m}$  ID PicoTip fused silica tubing (New Objectives, Woburn, MA) packed with C-18 material on a length of 8-9cm) by a gradient developed from 5% [v/v] formic acid to 80% [v/v] acetonitrile, 0.2% [v/v] formic acid in water at a flow rate of 200-500 nl/min.

#### *Tandem Mass Spectrometry: Instrument Setting for the LTQ-Orbitrap*

In general, tandem mass spectrometry (MS/MS) involves first the measurement of the masses of intact tryptic peptides, resulting in the acquisition of a full mass spectrum (the precursor ion spectrum). Because the mass of a peptide is often not unique and sufficient for unambiguous identification, further fragmentation is required to gain structural information such as the peptide's amino acid sequence. Selected precursor ions are singularly transferred to a collision-induced dissociation (CID) cell, where they undergo a forced but controlled fragmentation. Peptide fragments are generated from the breakages of peptide bonds between amino acids. Two types of fragments are created: one resulting from the fragmentation of the peptide from the amino terminus and the other from the fragmentation beginning at the carboxyl terminus; both ladders are represented in the resulting MS/MS spectrum. The way each amino acid fragments from the peptide is unique, and therefore structural information can be obtained. This amino acid peptide sequence can then be searched against a known proteome database and the homologue identity of the protein can be discussed. This leads to the acquisition of a fragment spectrum (product ion spectrum), thus providing a finger print characteristic of that peptide's amino acid sequence. For more information, see (Marcotte, 2007) for a review.

Orbitraps are ion trapping spectrometers which are usually coupled with the ion source (Yates *et al.*, 2009), in this case electron spray ionisation. Here, a hybrid mass spectrometer, the LTQ-Orbitrap, was used. This type of spectrometer is useful for protein identification, quantification, and PTM identification (Yates *et al.*, 2009), and can operate in a parallel fashion: the LTQ carries out fragmentation reactions (including CID), and the Orbitrap simultaneously acquires the MS full scans of these peptides (Yates *et al.*, 2009). Orbitrap mass analysers are particularly useful instruments, as they have a high resolution (up to 150,000), high mass accuracy (2-5 ppm), a mass-to-charge range of 6000, and a dynamic range greater than  $10^3$  (see Yates *et al.*, 2009 for review). The LTQ-Orbitrap hybrid instrument has the advantage of both the high resolution and mass accuracy of the Orbitrap, and the speed and sensitivity of the LTQ (Yates *et al.*, 2009). Full MS in a mass range between  $m/z$  300-2000 was performed in the Orbitrap mass analyser with a resolution of 60,000 at  $m/z$  400 and an AGC target of  $5e5$ . Preview mode for FTMS master scan was

enabled to generate precursor mass lists. The strongest five signals were selected for collision induced dissociation (CID)-MS/MS in the LTQ ion trap at a normalised collision energy of 35% using an AGC target of 2e4 and one microscan. Dynamic exclusion was enabled with two repeat counts during 30 sec. and an exclusion period of 180 sec. Exclusion mass width was set to 0.01.

### *Data Analysis*

*De novo* peptide sequencing is the process of determining the amino acids in each trypsin-created peptide fragment of the protein of analysed; thus identifying the protein and any PTMs present. This is achieved by identifying the peptides in the sample of interest by comparing the resulting experimental spectra/ peptide masses against a database of known mass spectra (Aebersold and Mann, 2003, Marcotte, 2007). However, accurately identifying amino acids by their masses is the main computational challenge of MS-based proteomics (Marcotte, 2007), as some amino acids have identical masses (e.g. Leucine and Isoleucine). Therefore, it helps to also use a sequence homology application in tandem, thus limiting the amounts of false-positives received; this, however, is limited to sequences which have already been documented on a database.

Here, probability based matching approach was used; this technique finds the best matches that meet the pre-defined minimum criteria for statistical significance (see Marcotte, 2007), by comparing the calculated fragments from peptide sequences in the database with the observed data MS peaks. An ion score is calculated from this comparison which gives the statistical significance of the match between the experimental and expected data (see Aebersold and Mann, 2003). Filtering criteria based upon database search scores, and other available data, can be applied to identify correct from incorrect peptide assignments. Specifically, MS/MS data were scanned against the subset of the NCBI non-redundant protein sequence database (downloaded in August 2010) using the MASCOT search engine (<http://www.matrixscience.com>), with mollusca as the taxon group. The search was set up for full tryptic peptides with a maximum of three missed cleavage sites. The amino acids most commonly modified are lysine (K) and arginine (R), hence the focus on those for their modifications. Acetyl (K), methyl (K), methyl (R), di-methyl (K), di-methyl (R), tri-methyl (K), Carboxyamidomethyl cysteine (C), deamidation (NQ), oxidation (M) were included as variable modifications. The latter three modifications are artificial modifications resulting from sample handling. The precursor mass tolerance threshold was 10 ppm and the maximum fragment mass error 0.8 Da.

### *Criteria of Data for Inclusion*

For each histone, the MASCOT-generated suggested ion score was used, as this number represented the hits with  $p < 0.05$ . This number varied with each different histone and each snail analysed, so was changed accordingly. This more stringent criterion reduced the number of false identifications in the data set. For histones with more than one significant hit, as defined by the MASCOT search engine, the expected value (e-value) was used to determine which sequence was used and determined to be the closest sequence match. The e-value is a parameter that describes the number of hits generated by chance when searching a database of a particular size. It decreases exponentially as the ion score of the match increases. It is essentially giving the probability of multiple sequence matches. Here, the e-value was used as a statistical validation that the sequence matched is a unique identification, rather than an ambiguous match. The general cut-off value of 0.05 was used as a sign of significance. The ion score was also taken into account, with a combination of high ion score and low e-value being markers of high sequence match.

For peptide sequences with multiple matches, the match with the highest ion score was included, as each snail histone type was only included once for each sequence in the final results. It was also necessary to confirm the matches MASCOT provided were indeed true positive matches. Each modified amino acid identified was confirmed as a true unambiguous match by looking at its matched sequence and determining whether it had matched amino acids on either side (on both the b- and y-ion series) of the amino acid of interest. These matched amino acids indicate that MASCOT had indeed confirmed the addition of the modification on the amino acid and thus matched the following amino acids on the sequence accordingly. Conversely, an ambiguous match had little to none confirmed matches following the modified amino acid. Furthermore, examining the spectra of matched amino acid sequences also helped separate unambiguous from ambiguous matches, as unambiguous matches of modified amino acids showed up as distinct peaks with a clear shift in  $m/z$  when compared to their uninfected counterpart.

As protein loading for each sample was varied, due to differences in snail tissue size, potential differences in protein abundance in the snail tissue, and limitations of the protein extraction method, some amino acid PTMs were absent from the MASCOT analysis. To detect whether these modifications were present, but in too low concentrations to be detected by MASCOT, manual searching of the raw data was necessary. This involved using the  $m/z$  value of a matched amino acid in a different snail tissue sample to search for the same peak in the sample which was missing. For example, if a methylation was detected on H3K9 in the

*Philophthalmus* sp. infected samples but missing in the uninfected gonadal tissue samples, the m/z number from one of the *Philophthalmus* sp. samples would be used, e.g. 664.3669, to search for a peak in the uninfected gonads sample. The presence of a peak indicates that the modification is present in the sample, but the concentrations were below the detecting threshold of the MASCOT program. The peak found must also be evaluated to determine whether it is a clear, definite peak, or merely background noise. On the results tables  $\div\emptyset$  denotes amino acid modifications which were found through manually searching the raw data, and  $\div\emptyset$  denotes modifications which could not be found through a manual search but cannot be definitively determined as an absent modification due to the uncontrollable variations in protein loading. For the colour sequenced results diagrams, the colours used to signify the modification type on the amino acids were assigned based on a hierarchy system illustrated in Figure 6.

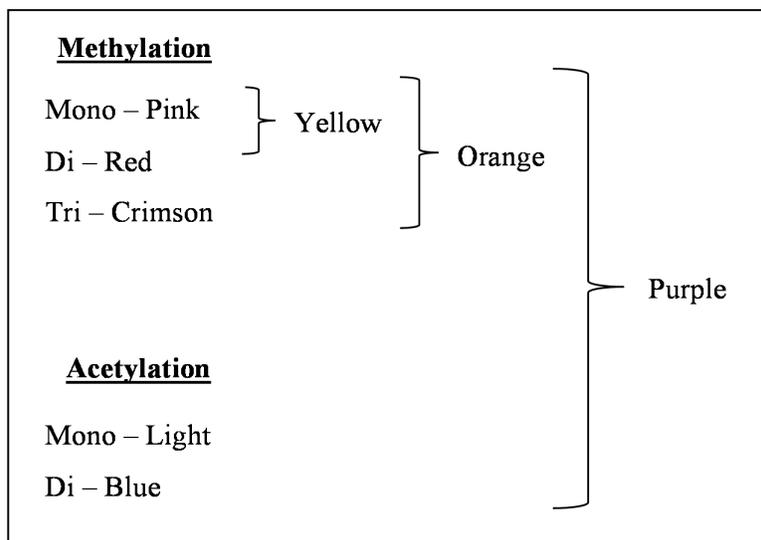


Figure 6. Colour configuration of modifications on amino acids.

### 4.3 Results

Table 2. Detected post-translational modifications on histone H2A from five samples: uninfected whole *Zeacumantus subcarinatus* snails, uninfected somatic tissue, uninfected gonadal tissue, *Z. subcarinatus* snails infected with *Philophthalmus* sp. and *Z. subcarinatus* snails infected with *Maritrema novaezealandensis*. Histone sequences were matched with the closest sequenced species as determined by the MASCOT program. Modified amino acids are bolded in the peptide sequences. Bullets (É) indicate how many of the three samples the specific modification was detected in according to MASCOT analysis. A plus sign (+) denotes modifications which were not detected by MASCOT, but were found to be present upon manual searching.

Histone (Species match)	Modified Amino Acid	Uninfected Whole	Uninfected Somatic Tissue	Uninfected Gonads	<i>Philophthalmus</i> sp. Infected	<i>M. novaezealandensis</i> <i>Infected</i>
<b>Histone H2A</b> ( <i>Mytilus edulis</i> )						
R.AGLQFPVGR.I	R29	+	+	+	methyl-arginine É	+
R.KGNYAER.V	R42	di-methyl-arginine É	+	+	+	+

Histone H2A does not appear to be very heavily methylated (table 2). Although manual examination of the raw data identified the presence of methylation of arginine 29 (H2AR29) and di-methylation of arginine 42 (H2AR42) in all samples, their presence was mostly too low to be detected by the MASCOT program (table 2).

Table 3. Detected post-translational modifications on histone H2B from five samples: uninfected whole *Zeacumantus subcarinatus* snails, uninfected somatic tissue, uninfected gonadal tissue, *Z. subcarinatus* snails infected with *Philophthalmus* sp. and *Z. subcarinatus* snails infected with *Maritrema novaezealandensis*. Histone sequences were matched with the closest sequenced species as determined by the MASCOT program and includes matches in other sequenced species. Modified amino acids are bolded in the peptide sequences. Bullets (É) indicate in how many of the three samples the specific modification was detected in according to MASCOT analysis. A plus sign (+) denotes modifications which were not detected by MASCOT, but were found to be present upon manual searching. A dash (-) denotes modifications which could not be detected by MASCOT or by manual search, but cannot be definitively determined as absent due to potential experimental errors. Note: The sequence histone H2B matched both H2B *Mytilus californianus* and H2B Gonadal with an equal top protein score, though the H2B gonadal sequence was used as this sequence matched more of the data set, and hence, the H2B match was likely incorrect; though the unique match in H2B *M. californianus* has been included.

Histone (Species match)	Modified Amino Acid	Uninfected Whole	Uninfected Somatic Tissue	Uninfected Gonads	<i>Philophthalmus</i> sp. Infected	<i>M. novaezealandensis</i> Infected
<b>H2B Gonadal</b>						
K.AGKAKAAR.S	K15	acetyl-lysine É	acetyl-lysine É	+	acetyl-lysine ÉÉ	+
	K17	acetyl-lysine É	acetyl-lysine É	+	acetyl-lysine ÉÉ	+
	R20	di-methyl-arginine É	di-methyl-arginine É	+	di-methyl-arginine ÉÉ	+
K.VLKQVHPDTGVS SK.A	K54	methyl-lysine ÉÉ	methyl-lysine É	methyl-lysine ÉÉ	methyl-lysine ÉÉÉ	methyl-lysine ÉÉÉ
K.AMSIMNSFVNDI FER.I	R69	di-methyl-arginine ÉÉÉ	di-methyl-arginine É	di-methyl-arginine ÉÉ	di-methyl-arginine ÉÉÉ	di-methyl-arginine ÉÉÉ
R.LLLPGELAKHAV SEGTK.I	K105	+	+	+	+	acetyl-lysine É
<b>H2B (<i>Mytilus edulis</i>)</b>						
K.VLRQVHPDTGVS SK.A	K56	methyl-lysine É	+	+	methyl-lysine É	+
K.AVTKYTSSK.-	K124	+	+	-	+	methyl-lysine É
<b>H2B (<i>M. californianus</i>)</b>						
R.STITSREVQTAVR LLLPGELAK.H	R91	+	-	-	methyl-arginine É	+

**U Whole/ U Somatic/ P-infected**

1 MPPKVSSKGA KKAG**KAKAAR** SGDKKRKRRR KESYSIYYK VLKQVHPDTG  
51 VSS**K**AMSIMN SFVNDIF**ERI** AAEASRLAHY NKRSTITSRE IQTAVRLLLLP  
101 GELAKHAVSE GTKAVTKYTS SK

**U Gonads**

1 MPPKVSSKGA KKAGKAKAAR SGDKKRKRRR KESYSIYYK VLKQVHPDTG  
51 VSS**K**AMSIMN SFVNDIF**ERI** AAEASRLAHY NKRSTITSRE IQTAVRLLLLP  
101 GELAKHAVSE GTKAVTKYTS SK

**M-infected**

1 MPPKVSSKGA KKAGKAKAAR SGDKKRKRRR KESYSIYYK VLKQVHPDTG  
51 VSS**K**AMSIMN SFVNDIF**ERI** AAEASRLAHY NKRSTITSRE IQTAVRLLLLP  
101 GELAKHAVSE GTKAVTKYTS SK

KEY	
	Mono-acetyl
	Mono-methyl
	Di-methyl

Figure 7. Comparison of H2B sequences matched by MASCOT program between samples from whole uninfected *Zeacumantus subcarinatus* snails (U Whole), uninfected *Z. subcarinatus* somatic tissue (U Somatic), uninfected *Z. subcarinatus* gonadal tissue (U gonads), *Philophthalmus* sp. infected (P-infected) *Z. subcarinatus* snails, and *Maritrema novaezealandensis* infected (M-infected) *Z. subcarinatus* snails. Amino acids in bold identify sequences which have been matched by MASCOT to homologous sequences in a sequenced organism, those not bolded are sequences unique to *Z. subcarinatus*. Modifications on amino acids are indicated by colour shown in the key. Differences between samples indicated by underlining of affected amino acid.

There appear to be no overall differences in the pattern of PTMs on histone H2B between the samples analysed (table 3 and fig. 7). The presence of PTMs on histone H2B is constant between the various samples with a noticeable lowered presence of modifications detected in the samples from *Z. subcarinatus* uninfected gonad tissue. Again, due to difficulties in ensuring even protein loading, any differences detected in *Z. subcarinatus* uninfected gonad tissue cannot be definitively ruled as a lack of modification. The modification of lysine 54 (H2BK54) and arginine 69 (H2BR69) are determined by MASCOT as present in all samples, with the di-methylation of arginine 69 being the most consistently present modification of those detected on histone H2B, having a detection rate of 12 out of 15 samples run (table 3 and fig. 7).

*Philophthalmus* sp. infected samples have the highest presence of PTMs on lysine 15 (H2BK15) and 17 (H2BK17) and arginine 20 (H2BK20), and also the only MASCOT-detected presence of methylation on arginine 91 (H2BR91) on the matched sequence from *Mytilus californianus* (table 3 and fig. 7). *Maritrema novaezealandensis*-infected samples have the highest presence of acetylation on lysine 105 (H2BK105), and the only MASCOT-detected presence of methylation on lysine 124 (H2BK124) on the matched sequence from histone H2B *Mytilus edulis* (table 3 and fig. 7).

Table 4. Detected post-translational modifications on histone H3 from five samples: uninfected *Zeacumantus subcarinatus* snails, uninfected somatic tissue, uninfected gonadal tissue, *Z. subcarinatus* snails infected with *Philophthalmus* sp. and *Z. subcarinatus* snails infected with *Maritrema novaezealandensis*. Histone sequences were matched with the closest sequenced species as determined by the MASCOT program and includes matched modifications in other sequenced species. Modified amino acids are bolded in the peptide sequences. Bullets (É) indicate in how many of the three samples the specific modification was detected in according to MASCOT analysis. A plus sign (+) denotes modifications which were not detected by MASCOT, but were found to be present upon manual searching. A dash (-) denotes modifications which could not be detected by MASCOT or by manual search, but cannot be definitively determined as absent due to potential experimental errors.

Histone (Species match)	Modified Amino Acid	Uninfected Whole	Uninfected Somatic Tissue	Uninfected Gonads	<i>Philophthalmus</i> sp. Infected	<i>M. novaezealandensis</i> Infected
<b>Histone H3</b>						
<i>(Aplysia californica)</i>						
R.KSTGGKAPR.K	K10	methyl-lysine É	+	+	+	+
		di-methyl-lysine É	di-methyl-lysine ÉÉ	+	di-methyl-lysine É	di-methyl-lysine ÉÉ
	K15	acetyl-lysine ÉÉ	acetyl-lysine É	acetyl-lysine ÉÉ	acetyl-lysine ÉÉ	acetyl-lysine É
	K10 and K15	methyl-lysine & acetyl-lysine ÉÉÉ	methyl-lysine & acetyl-lysine ÉÉ	methyl-lysine & acetyl-lysine É	methyl-lysine & acetyl-lysine ÉÉÉ	methyl-lysine & acetyl-lysine ÉÉÉ
		di-methyl-lysine & acetyl-lysine ÉÉÉ	di-methyl-lysine & acetyl-lysine ÉÉÉ	di-methyl-lysine + acetyl-lysine ÉÉÉ	di-methyl-lysine + acetyl-lysine ÉÉÉ	di-methyl-lysine + acetyl-lysine ÉÉÉ
	2 acetyl-lysine É	2 acetyl-lysine ÉÉ	+	2 acetyl-lysine ÉÉÉ	2 acetyl-lysine ÉÉÉ	
R.KQLATK.A	K19	acetyl-lysine É	acetyl-lysine É	+	+	+
R.KQLATKAAR.K	K24	acetyl-lysine ÉÉÉ				
	K19 and K24	2 acetyl-lysine ÉÉ	2 acetyl-lysine É	+	2 acetyl-lysine ÉÉ	2 acetyl-lysine É

R. KSAPATGGVKKPH R.K	K28	methyl-lysine É	methyl-lysine É	+	methyl-lysine Ę	methyl-lysine É
		di-methyl-lysine Ę	di-methyl-lysine Ę	di-methyl-lysine Ę	di-methyl-lysine Ę	di-methyl-lysine Ę
		tri-methyl-lysine Ę	tri-methyl-lysine É	+	tri-methyl-lysine Ę	tri-methyl-lysine Ę
	K37	di-methyl-lysine É	di-methyl-lysine É	+	di-methyl-lysine É	di-methyl-lysine É
	K28 and K37	di-methyl-lysine & methyl-lysine Ę	di-methyl-lysine & methyl-lysine É	di-methyl-lysine & methyl-lysine É	di-methyl-lysine & methyl-lysine Ę	di-methyl-lysine & methyl-lysine Ę
		+	tri-methyl-lysine & methyl-lysine É	+	+	+
R.EIAQDFKTDLR.F	K80	methyl-lysine É	methyl-lysine Ę	+	methyl-lysine Ę	methyl-lysine É
R.FQSSAVMALQEA SEAYLVGLFEDTNL CAIHAK.R	K116	-	-	-	methyl-lysine Ę	-
<b>H3L-like histone</b> ( <i>Sepia officinalis</i> ) K.STELLVRK.L	R49	methyl-arginine É	+	+	methyl-arginine Ę	+
<b>Histone H3</b> R.FQSAAGALQEA SEAYLVGLFEDTNL CAIHAK.R	K116	-	-	-	methyl-lysine É	-

<b>U Whole/ U Somatic/M</b>		
<b>1</b>	MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR	
<b>51</b>	EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSSAV MALQEASEAY	
<b>101</b>	LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA	
<b>U Gonads</b>		
<b>1</b>	MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR	
<b>51</b>	EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSSAV MALQEASEAY	
<b>101</b>	LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA	
<b>P</b>		
<b>1</b>	MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR	
<b>51</b>	EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSSAV MALQEASEAY	
<b>101</b>	LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA	
		<b>KEY</b>
		Mono-acetyl
		Mono-methyl
		Di-methyl
		Mono + Di-methyl
		Mono + Di + Tri-methyl
		Acetyl + Methyl

Figure 8. Comparison of *Aplysia californica* sequences matched by MASCOT program between histone H3 samples from whole uninfected *Zeacumantus subcarinatus* snails (U Whole), uninfected *Z. subcarinatus* somatic tissue (U Somatic), uninfected *Z. subcarinatus* gonadal tissue (U gonads), *Philophthalmus* sp. infected (P-infected) *Z. subcarinatus* snails, and *Maritrema novaezealandensis* infected (M-infected) *Z. subcarinatus* snails. Amino acids in bold identify sequences which have been matched by MASCOT to homologous sequences in *A. californica*, those not bolded are sequences unique to *Z. subcarinatus*. Modifications on amino acids are indicated by colour shown in the key. Differences between samples are indicated by underlining of affected amino acid.

There appear to be no overall differences in the pattern of PTMs on histone H3 between the samples analysed (table 4 and fig. 8). The presence of PTMs on histone H3 is constant between the various samples with, again, a lower presence of modifications detected by MASCOT in the samples from *Z. subcarinatus* uninfected gonadal tissue. As mentioned, due to difficulties in ensuring even protein loading, it is not possible to make any definitive conclusions from the greatly different results of uninfected gonadal tissue. The acetylation of lysine 24 (H3K24) has been identified by MASCOT as the modification with the highest level of presence, being present in all samples run (table 4 and fig. 8).

Of note, one uninfected whole *Z. subcarinatus* sample showed the only MASCOT-detected methylation on lysine 10 (H3K10), and both uninfected whole samples and uninfected somatic tissue samples showed the only MASCOT-detected acetylation on lysine 19 (H3K19; table 4 and fig. 8). One uninfected somatic tissue sample possessed the only MASCOT-detected combination of tri-methylation and methylation on lysine 28 (H3K28) and 37 (H3K27; table 4 and fig. 8). *Philophthalmus* sp. infected samples possessed the only MASCOT-detected methylation of lysine 80 (H3K80) and lysine 116 (H3K116) on the matched sequence from histone H3 (table 4 and fig. 8).

Table 5. Detected post-translational modifications on histone H4 from five samples: uninfected *Zeacumantus subcarinatus* snails, uninfected somatic tissue, uninfected gonadal tissue, *Z. subcarinatus* snails infected with *Philophthalmus* sp. and *Z. subcarinatus* snails infected with *Maritrema novaezealandensis*. Histone sequences were matched with the closest sequenced species as determined by the MASCOT program. Modified amino acids are bolded in the peptide sequences. Bullets (É) indicate in how many of the three samples the specific modification was detected in according to MASCOT analysis. A dash (-) denotes modifications which could not be detected by MASCOT or by manual search, but cannot be definitively determined as absent due to potential experimental errors. Note: Despite there being many modifications on R24 they were considered false-positive matches by Mascot and are therefore not included in the results.

Histone Sequence (Species match)	Modified Amino Acid	Uninfected Whole	Uninfected Somatic Tissue	Uninfected Gonads	<i>Philophthalmus</i> sp. Infected	<i>M. novaezealandensis</i> Infected
<b>H4</b> <i>(Mytilus edulis)</i> K.GG <b>K</b> GLG <b>K</b> GGAK R.H	K9	acetyl-lysine ÉÉ	acetyl-lysine É	-	acetyl-lysine É	acetyl-lysine É
	K13 and K17	2 acetyl-lysine É	2 acetyl-lysine É	-	2 acetyl-lysine ÉÉ	2 acetyl-lysine É
	K9 and K13 and K17	3 acetyl-lysine É	3 acetyl-lysine É	-	3 acetyl-lysine ÉÉ	3 acetyl-lysine ÉÉ
R.KVLRDNIQG <b>I</b> TKP AIR.R	K21	di-methyl-lysine ÉÉÉ	di-methyl-lysine É	di-methyl-lysine ÉÉÉ	di-methyl-lysine ÉÉÉ	di-methyl-lysine ÉÉ
R.ISGLIYE <b>E</b> TR.G	R56	di-methyl-arginine É	di-methyl-arginine É	di-methyl-arginine É	di-methyl-arginine ÉÉ	di-methyl-arginine É

<b>U Somatic/ U Whole/ P-infected/ M-infected</b>		<table border="1"> <thead> <tr> <th colspan="2">KEY</th> </tr> </thead> <tbody> <tr> <td style="background-color: #00B0F0;"></td> <td>Mono-acetyl</td> </tr> <tr> <td style="background-color: #FF0000;"></td> <td>Di-methyl</td> </tr> </tbody> </table>	KEY			Mono-acetyl		Di-methyl
KEY								
	Mono-acetyl							
	Di-methyl							
1	MSGRGKGGK <u>G</u> LGKGGAKRHR <b>K</b> VLRDNIQGI TKPAIRRLAR RGGVKRISGL							
51	IYEETRGVLK VLENVIRDA VTYTEHAKRK TVTAMDVVYA LKRQGRTLYG							
101	FGG							
<b>U Gonads</b>								
1	MSGRGKGGK <u>G</u> LGKGGAKRHR <b>K</b> VLRDNIQGI TKPAIRRLAR RGGVKRISGL							
51	IYEETRGVLK VLENVIRDA VTYTEHAKRK TVTAMDVVYA LKRQGRTLYG							
101	FGG							

Figure 9. Comparison of *Mytilus edulis* sequences matched by MASCOT program between histone H4 samples from whole uninfected *Zeacumantus subcarinatus* snails (U Whole), uninfected *Z. subcarinatus* somatic tissue (U Somatic), uninfected *Z. subcarinatus* gonadal tissue (U gonads), *Philophthalmus* sp. infected (P-infected) *Z. subcarinatus* snails, and *Maritrema novaezealandensis* infected (M-infected) *Z. subcarinatus* snails. Amino acids in bold identify sequences which have been matched by MASCOT to homologous sequences in *M. edulis*, those not bolded are sequences unique to *Z. subcarinatus*. Modifications on amino acids are indicated by colour shown in the key. Differences between samples are indicated by underlining the affected amino acid.

There appear to be no overall differences in the pattern of post-translational modifications (PTMs) on histone H4 between the samples analysed (table 5 and figure 9 ). The presence of PTMs on histone H4 were constant between the various samples with the exception of samples from the uninfected *Z. subcarinatus* gonadal tissue. Again due to the inability to load protein evenly, the differences in uninfected *Z. subcarinatus* gonadal tissue cannot be definitively ruled as a lack of modification. The modifications on both lysine 21 (H4K21) and arginine 56 (H4R56) were detected in all samples, with di-methylation of lysine 21 being the most consistently present modification of those detected on histone H4, with a detection rate of 12 out of 15 samples (table 5 and figure 9). Of note, uninfected whole *Z. subcarinatus* samples have the highest presence of acetylation on lysine 9 (H4K9), *Philophthalmus* sp.-infected samples have the highest presence of acetylation on the combination of lysine 13 (H4K13) and 17 (H4K17), and acetylation on the combination of lysine 9, 13 and 17 has the highest presence in the *Philophthalmus* sp. and *M. novaezealandensis*-infected samples (table 5 and fig. 9).

#### 4.4 Discussion

The current study found no overall differences in the pattern of global histone post-translational modifications between uninfected *Zeacumantus subcarinatus* snails and snails infected with *Philophthalmus* sp. or *Maritrema novaezealandensis* parasites; specifically, no modifications unique to parasitised or unparasitised hosts were found. However, there appear to be disparities in the strengths of these modifications between samples as indicated by their varying levels, suggesting variations in quantity. The most heavily modified histones were H3 and H4, which naturally form a heterodimer (Wolffe and Pruss, 1996). This may be due to the composition of histone H3, consisting of 38 amino acids with 19 of these being potential modification sites; this results in a high density of PTMs on H3 (Villar-Garea and Imhof, 2006), with a potentially similar structure of H4. The lysine amino acids on the histone tails of histones H3 and H4 are also known to be the major targets of PTMs (Rice and Allis, 2001). The findings of the current study indicate that either (1) the parasitic infection is having no effect on its host's histone PTMs, or (2) parasitic infection is causing targeted changes at specific histone PTMs and therefore not causing a detectable global change.

##### *Histone PTM Quantification and Patterns*

The precise regulatory roles of histone PTMs mean that any major whole scale genome changes in histone PTMs are likely to be detrimental; indeed, such dramatic changes are usually associated with disease, such as cancer (Seligson *et al.*, 2005). Small, targeted changes in the patterning of histone PTMs appear to be the usual factors which elicit observable phenotypic outcomes, for example methylation of different lysine residues on the same histone tail can result in contrasting biological outcomes, namely transcriptional activation and silencing (Rice and Allis, 2001). Though the current study was able to broadly detect the presence or absence of PTMs on histones, the levels of these modifications in the various samples could not be quantified due to logistical constraints. However, there were varying strengths of the detected modifications across the samples, with some modifications appearing in almost all of the samples analysed. Indeed, the integrity of the histone pattern is likely to be an important aspect of normal genome function, with any changes to this pattern (absences or additional PTMs) likely to lead to disease, as it does in DNA methylation patterning (reviewed in Liu *et al.*, 2010). For instance, euchromatic chromatin is affected by euchromatic modification patterns which regulate transcription in a binary on/off configuration; the absence of any one of the essential modifications results in the chromatin

remaining in the silent state, indicating an ‘all or nothing’ scenario (Schübeler *et al.*, 2004). Following this hypothesis, varying levels of any one of the euchromatic modifications can result in changes in the ratio of transcriptionally active chromatin in a genome. Similarly, changes in the level of an individual modification can elicit negative effects. For example, a stronger level of H3K9 trimethylation is negatively correlated with prognosis in patients with gastric adenocarcinoma, possibly due to increasing the severity of the cancer and the likelihood of tumour recurrence (Park *et al.*, 2008). These examples demonstrate the importance of quantifying the level of histone PTMs when studying changes in global modification level. The current study encountered difficulties with the *Z. subcarinatus* gonadal tissue which interfered with any meaningful quantitative results which could have been obtained from the samples. The difficulties encountered with protein loading could be remedied by introducing a standard level of a known substance into the samples as a comparative tool; this was logistically not practical within the constraints of the current study, but should be implemented in future studies of this nature.

#### *Histone Code Hypothesis*

The precise pattern of histone PTMs is of known importance in the field of chromatin epigenetics. A current hypothesis, dubbed the ‘Histone Code’ theory, proposes that the combined effects of multiple specific histone modifications, both the type and number, act cooperatively or antagonistically to dictate a particular biological outcome (see Jenuwein and Allis, 2001, Berger, 2002, Peterson and Laniel, 2004, Smith and Denu, 2009). The hypothesis suggests that the various patterns of activation and repression due to PTMs spread across many histone tails on the chromatin can combine to elicit definitive biological outcomes. The hypothesis hinges on the association of histone tail modifications with effector proteins which act to sustain a specific chromatin structure or prompting gene activation or repression (reviewed in Stewart *et al.*, 2005). In accordance with this hypothesis, each histone PTM can affect the strength of the biological outcome as each individual PTM has an effect on the resulting outcome.

#### *Expansion upon Histone Code Theory*

Although no distinct pattern of histone modifications can be identified in the samples analysed in the current study, a recent study by Filion *et al.* (2010) expands upon the ‘Histone Code’ hypothesis by finding multiple states in activation and inactivation are induced

according to the effector proteins recruited by specific histone modification patterns. These effector proteins require specific histone PTMs as binding sites, PTMs therefore act as the positive- or negative-acting modifiers which recruit and provide a binding platform for the protein complexes required for gene expression. Thus, a finer classification of chromatin transcriptional state is required rather than the simple division of heterochromatin and euchromatin. The *Drosophila melanogaster* genome is now thought to be divided into five principal chromatin types, distinguished by their distinct regulatory functions and transcriptional state, which is determined by their specific histone PTMs and the associated effector protein complexes they recruit (Filion *et al.*, 2010). Additionally, the five types of chromatin also differ in many other characteristics, including transcriptional activity, histone modifications, biochemical properties, and replication timing, among others (Filion *et al.*, 2010). These findings re-affirm the importance of histone PTM quantification and expand upon the hypothesis of the Histone Code by suggesting that the composition and quantity of specific histone PTMs contribute to the resulting type of chromatin and the transcriptional state of associated genes.

### *Conclusions*

Though the current study found no whole-scale differences in the pattern of global histone modifications between uninfected *Z. subcarinatus* snails and those infected with *Philophthalmus* sp. and *M. novaezealandensis* trematodes, distinct differences in the strengths of the modifications were detected, suggesting they vary in quantity. Variations in the quantity of each PTM are an important aspect to consider as they can change the PTM patterning, which potentially affects the ratio of transcriptionally active chromatin in a genome and what type of effector proteins are recruited. Such chromatin changes in have the potential to elicit significant downstream effects on the resulting biological outcome. Though the current study was unable to accurately quantify changes of each histone PTM, it is clear that it is a significant defining feature in gene expression and resulting phenotypic outcomes. Such subtle changes in histone modification patterns, rather than large whole-scale changes, which often lead to disease, are more likely to be behind the observed parasite-induced phenotypic changes in the host.

## **5 General Discussion**

Though clear morphological changes occur in the shell of *Zeacumantus subcarinatus* following infection, the present study found of evidence for changes in DNA methylation patterns or histone methylation levels in the mud snail, *Z. subcarinatus*, associated with parasitic infection by the trematodes *Philophthalmus* sp. or *Maritrema novaezealandensis*. The findings of the present study indicate that either (1) the parasitic infection is having no epigenetic effect on its host, or (2) parasitic infection is causing targeted changes at specific genes and histones, therefore not causing a detectable epigenetic global change. The observed phenotypic changes in the host are possibly induced by parasite-induced changes in specific DNA methylation patterning in conjunction with changes in the levels of histone post-translational modifications. However, caution is required when interpreting the results of the present study as they are based on a rather small number of samples (12 organisms in total, 3 of each infection status).

Nevertheless, this research opens the door for further investigation into the possible effects of changes in DNA methylation patterning and small variations in the level of each histone post-translational modification (PTM). Indeed, small epigenetic variations are known to have major downstream effects due to the regulatory role of epigenetic systems on gene expression (reviewed in Liu *et al.*, 2010) Any changes in the balance and pattern of epigenetic modifications either at the level of DNA methylation or histone modifications will likely impact the resulting phenotype, as these two systems interact to propagate a self-reinforced transcriptional state (reviewed in Quina *et al.*, 2006). The interaction of the two epigenetic systems and the resulting transcriptional state is facilitated by effector proteins, which are recruited by both systems (reviewed in Bachman *et al.*, 2001 and Kouzarides, 2002). Though it has yet to be formally studied, this highly reactive system of epigenetic gene transcriptional regulation is likely to have effects in shell formation, given that shell formation is under strong biological and genetic control (Cook, 1965, Jackson *et al.*, 2010).

### *Shell formation*

The Molluscan shell is of ectodermic origin and is secreted by the mantle at intervals throughout the organism's lifetime (Ruppert and Barnes, 1994, Marin and Luquet, 2004). Shell formation is strongly biologically controlled: spatial mapping of gene expression profiles on the mantle orders the development of the growing shell (Jackson *et al.*, 2006), hundreds of mantle-secreted proteins (creating an organic shell matrix) strictly regulate crystal formation, structure and density (Belcher *et al.*, 1996, Marin and Luquet, 2004). Genes encoding these shell proteins comprise more than 25% of the genes expressed in the mantle of

the vetigastropod *Haliotis asinina* (Jackson *et al.*, 2006). Furthermore, 85% of the secretome of *H. asinina* comprises novel proteins, with only 19% of these having homologues in only one related species, suggesting that the complex proteins which direct the highly structured development of the molluscan shell are encoded by rapidly evolving genes (Jackson *et al.*, 2006). The shell colour and patterning of the snail *Cepaea nemoralis* are also shown to be under genetic control through cross-breeding studies which reveal the many phenotypic variations have different degrees of dominance (Cain *et al.*, 1960).

Despite the high degree of biological control in the shell formation process, there exists a degree of plasticity in Molluscan shell formation as evidenced by the susceptibility of the process to biotic and abiotic environmental influences. For example, shell thickness increases in several species in response to cues associated with the presence of a crab predator, and decreases in snails living in areas of colder water (reviewed in Trussell and Etter, 2001). Furthermore, *C. nemoralis* shell colour is known to be affected by environmental factors such as climate change (O go and Schilthuizen, 2012). Shell plasticity is not completely separate from genetic change; plasticity can alter the evolution of the genotype by influencing the adaptive responses of an organism through the target of selection, the phenotype (Trussell and Etter, 2001). Indeed, changes to any one of the transcriptional states of the genes which regulate the proteins of the organic shell matrix are likely to affect the crystal formation, and therefore affect the resulting shell composition; additionally, epigenetic changes to gene transcriptional states also have the potential to significantly increase the diversity of the organic matrix (Jackson *et al.*, 2006).wound\

Trematode parasitic infection is a strong environmental stimulus which results in a decreased shell width in the mud snail, *Zeacumantus subcarinatus* (Hay *et al.*, 2005). Though the current study found no epigenetic differences between infected and uninfected *Z. subcarinatus*, given that the molluscan shell is under genetic control and the reactivity of the epigenetic gene regulatory system suggests a link between parasitic infection and epigenetic changes in the host is plausible.

#### *Parasites affecting Gene Expression*

Though distinct differences in gene expression were not strongly evident between infected and uninfected snails in the current study, symbiotic relationships and parasitic infection have been shown to elicit changes in gene expression. The symbiont-induced genetic changes in the temperate sea anemone *Anthopleura elegantissima* parallel the findings of the

current study: no set of symbiosis-specific genes is responsible for controlling and regulating symbiosis, rather the alteration of the expression of a broad array of genes regulating various functional processes occurs (Rodriguez-Lanetty *et al.*, 2006). The resulting differential expression of 28 anemone host genes illustrates the complex effect of the symbiotic state on host gene expression. This suggests that symbiosis is maintained by inducing changes in existing pathways associated with metabolism and growth of the host, rather than by the manipulation of pathways unique to the symbiosis (Rodriguez-Lanetty *et al.*, 2006). Although the differences in the expression levels of genes could not be determined in the current study, the symbiont-induced changes in the anemone *A. elegantissima* provide evidence that such organisms can instigate changes at the level of gene expression and are worth exploring in other symbioses such as parasitism. Indeed, parasites are known to elicit changes to host gene expression in ways which are beneficial to the parasites themselves: parasitic plant bacteria are known to target host processes such as expression of defence genes, hormone signalling and programmed apoptosis (reviewed in Abramovitch and Martin, 2004). Additionally, helminth parasites have been found to stimulate apoptosis in non-infected host cells (reviewed in James and Green, 2004).

The current study investigated changes in gene expression in a snail host following infection by trematode parasites. Though no clear differences were detected between infected and uninfected snail hosts, trematode parasites indeed have the ability to induce broad changes in neuronal gene expression in snail hosts (Hoek *et al.*, 1997), which directly induces the differential expression of genes encoding neuropeptide precursors involved in the regulation of vital behavioural and physiological processes. Furthermore, the expression profiles of the transcripts tested infer that gene expression is constantly adjusted throughout infection in ways which increase the parasite's fitness. Parasite-induced changes in host gene expression are not limited to animal hosts. For instance, root-knot nematodes, *Meloidogyne* sp., are widely considered to be the most evolutionarily advanced and successful parasites, and induce the development of a novel cell type, giant cells, in the host's roots which express many genes not normally expressed in mature root cells (see Bird, 1996). Furthermore, the root-knot nematode also appears to cause the apparent absence of typical host wound or defence responses (Bird, 1996). This suppression of, or failure to, elicit host wound/defence responses are active throughout infection, and may explain why nematode-induced galls are more susceptible to fungal and bacterial infection.

Though no conclusive changes in DNA methylation and histone modifications following infection were evident in this study, it is likely that the observed phenotypic

changes in the host shell result from parasitic infection, as shell formation is under genetic control and parasites are known to have an effect on host physiology and gene expression. This avenue of research warrants further investigation, as parasites and their hosts are known to have significant direct effects on each other's gene make up and are thought to be engaged in an evolutionary arms-race. This arms-race is the basis of the Red Queen hypothesis (Lively *et al.*, 1990) which proposes that co-evolution in host-parasite systems is attributed to the maintenance of sexual reproduction and the maintenance of polymorphisms (reviewed in Thompson and Burdon, 1992). The concept of gene-for-gene coevolution in these systems suggests this leads to pairs of species interacting in very specific ways, for example, for every gene determining resistance in the host there is a corresponding specific gene for virulence in the parasite (reviewed in Thompson and Burdon, 1992). This concept suggests that parasitic interactions have had a significant role in driving evolution, though in a semi-indirect way, as the concept posits the host/parasite responds to specific changes each other's genetic strategies by developing a corresponding genetic counter-attack. The current study intended to take this idea one step further and investigate whether multicellular parasites can take a very offensive approach and directly modify the genetic expression of their multicellular host. The findings of the present study establish a broad foundation upon which further investigations can take a more focused approach.

#### *Recommendations for future directions*

As mentioned, an important next step in investigating parasite-induced epigenetic changes in hosts is to quantify changes in DNA methylations and histone PTMs, therefore establishing changes in epigenetic patterning which may occur in parasitised hosts. To make quantification possible, a standard amount of a known compound could be added to each sample run, so the resulting output can be compared to the standard. Pairing snails of the same infection status would help increase the amount of protein available for each comparison, as most proteins are present in low abundances and there is currently no polymerase chain reaction (PCR)-equivalent way of amplifying proteins (Herbert *et al.*, 2001). Moreover, protein identifications rely on matching the protein sequence obtained with homologous sequenced species available in the database, therefore limiting accurate protein identifications, and correspondingly quantifications. Though it was not possible in the current study, using a host species which has been comprehensively sequenced and which shows parasite-induced phenotypic alterations would allow more insights into the results obtained. The current study also initially intended to investigate whether parasite-induced epigenetic changes were the

cause of observed host behavioural changes in a different system, and whether any epigenetic changes were passed onto the next generation. Trematode-induced behavioural changes in an amphipod host were intended to be investigated, as amphipods are the second intermediate host and are not castrated in the chosen system. Though time limitations prevented this being possible, investigation into this dimension of parasite-induced host manipulation would garner interesting results into the mechanism behind changes in host behaviour.

### *Conclusions*

The current study determined that parasitic infection does not broadly affect host epigenetics. Specifically, there are no significant differences in the overall level of DNA methylation and the broad patterning of histone PTMs between uninfected gastropod hosts and those infected with a trematode. These findings fit with the current knowledge in this field which finds that epigenetic changes on such broad levels often lead to disease, as these epigenetic marks are important gene regulators. Though the current study offers no definitive answers about whether parasitic infection causes epigenetic changes in the host, and whether this is the mechanism behind parasite-induced host manipulation, the findings suggest that any parasite-induced epigenetic changes in their host are likely to be targeted at the level of DNA methylation patterning and changes at the individual quantity of each histone PTM, and therefore future studies should be focused at such a targeted level.

## **6 Additional Methodological Details**

## 6.1 Gel Recipes

### *Resolving Gel Recipe (15%)*

2.4ml 50% w/v Glycerol

5ml 30% degassed acrylamide

2.5ml Resolving Gel Buffer (1.5M Tris-HCL, pH 8.8)

27.23g Tris base

80ml deionised water

Adjust to pH 8.8 with 6N HCl. Bring total volume to 150ml with deionised water and store at 4°C.

100 µl 10% w/v SDS

50 µl 10% ammonium persulphate (w/v; ddH<sub>2</sub>O)

5 µl Tetramethylethylenediamine (TEMED)

### *Stacking Gel Recipe (15%)*

5.5ml deionized water

600 µl 50% w/v Glycerol

1.3ml 30% degassed acrylamide

2.5ml Stacking Gel Buffer (0.5M Tris-HCL, pH 6.8)

6g Tris base

60ml deionised water

Adjust to pH 6.8 with 6N HCl. Bring total volume to 100ml with deionised water and store at 4°C.

100 µl 10% w/v SDS

50 µl 10% ammonium persulphate (w/v; ddH<sub>2</sub>O)

10 µl Tetramethylethylenediamine (TEMED)

## 6.2 Buffer Recipes

### *Sample Buffer (SDS Reducing Buffer) Recipe*

1.875ml 0.5M Tris-HCL, pH 6.8

5ml Glycerol

2mg SDS powder

0.2mg bromophenol blue powder

Used deionized water to make solution up to 10ml

### *10x Electrode (Running) Buffer, pH 8.3*

30.3g Tris base

144.0g Glycine

10g SDS

Dissolve and bring total volume up to 1,000ml with deionised water. Do not adjust pH with acid or base. Store at 4°C. If precipitation occurs, warm at room temperature before use.

## 6.3 Stain Recipe

### *Colloidal Coomassie Stain*

c. 650 ml distilled water

100g ammonium sulfate

20ml of coomassie G-250 (5% solution dissolved in water)

30ml orthophosphoric acid

200ml ethanol

Add the ingredients in the order listed. Ensure the ammonium sulfate is dissolved before adding the coomassie G-250 solution to a final concentration of 0.1%. Bring up to 1000ml with additional deionised water. Store at room temperature with a tightly sealed lid.

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