Epigenetic factors affect the host:pathogen interaction between *Schistsoma mansoni* and *Biomphalaria glabrata*

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by

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Abstract

Schistosomiasis, also known as Bilharzia, is a neglected tropical disease (NTD) caused by the Schistosoma genus of trematode parasite and is estimated to affect 250 million people globally. The nucleus is a highly organised organelle with chromosomes and genes occupying distinct and reproducible locations. However, this spatial organisation is not fixed and different events such as differentiation, environmental stimuli, stress and replicative senescence can trigger genome reorganisation within nuclei. Previous work has already shown that when *Schistosoma mansoni* infects its obligate intermediate host *Biomphalaria glabrata*, a freshwater snail, it induces chromatin reorganisation resulting in subsequent upregulation of genes. *B. glabrata* genome organisation is more similar to mammalian than other invertebrates, meaning that there is potential for what can be discovered in the snail model to be applicable to the mechanism of infection in the human population.

The mechanisms that are responsible for inducing gene movement or chromatin reorganisation are poorly understood, but hypothesised to be partly as a result of epigenetic signalling. Histone methyl modification patterning within the nuclei of *B. glabrata* were investigated following several events known to induce or result in chromatin reorganisation, heat-shock, infection and ageing. Following comparisons between controls and experimental groups several changes in pattern distribution were identified. Infection of *B. glabrata* by *S. mansoni* H3K79me^3 showed significant alteration not replicated by heat-shock or ageing indicating that modification of H3K79me^3 is an important target for the parasite infection. As such a protocol was developed to further investigate the visual co-localisation of gene and histone modification signals.

To investigate the effects that potentially disrupting this induced chromatin reorganisation can cause, several drugs have been screened in the snail to assess their effect on subsequent changes in susceptibility. Susceptibility to infection was either assessed by one of two means. An absolute method scoring for complete resistance to infection. The second was counting the number of cercariae, the human infective stage of the *Schistosoma* lifecycle, that were shed from the snail. Drugs chosen were shown to either inhibit gene movement or target epigenetic factors that could be signalling for genome reorganisation to occur. Preliminary data have shown that affecting the acetylation within *B. glabrata* nuclei affects the snail's susceptibility to *S.mansoni* infection.

B. glabrata interphase genome organisation exhibits similarity to human nuclei, making it an excellent model organism for investigating the effects of genome reorganisation *in vivo* and as an invertebrate could be used to replace higher order models to reduce the use of animal models in research in line with the NC3R initatives. With a comparatively short lifespan of 12 months it is ideal for exploring, *in vivo*, ageing related changes to genome organisation. We have previously shown that gene movement and relocation to a new non-random location is possible within a short time period following a heat shock or an infection within juvenile snails. In 12 month aged snails it is demonstrated that significant genome reorganisation has occurred, with the *heat shock protein 70kDa* (*hsp70)* loci occupying a new non-random location within the nuclei and that neither heat-shock nor *S. mansoni* infection can induce gene relocation. Thus indicating not just significant changes to genome organisation but a potential loss of the mechanisms that are responsible for reorganisation of the chromatin.

Using fluorescent imaging techniques, alterations to histone markers, protein distribution and gene loci positioning within nuclei were investigated at varying ages within *B. glabrata*. This verifies work done in human senescent cells *in vitro* replicating changes to genome organisation and chromobility. As such this work presents *B. glabrata* as a new model for investigating the effects of ageing on nuclear organisation *in vivo*.

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Abbreviations

2D	Two Dimensional
3C	Chromosome Conformation Capture
3D	Three Dimensional
AA	Anacardic Acid
BB02	Wild-Type susceptible strain *B.glabrata* from Brazil
BAC	Bacterial Artificial Chromosome
BDM	2,3-Butanedione monoxime
Bge	*Biomphalaria glabrata* embryonic
bp	Base Pair
BS-90	Wild-Type resistant strain of *B. glabrata* from Brazil
BSA	Bovine Serum Albumin
CCF	Cytoplasmic Chromatin Fragment
ChIP	Chromatin Immunoprecipitation
CS	Cold Shock
Cy3	Cyanine 3
Cy5	Cyanine 5
DαG	Donkey anti-Goat
DαR	Donkey anti-Rabbit
DAPI	4, 6 di-amidino-2-phenylindole
dATP	Deoxyribose Adenosine Triphosphate
dH_2O	Distilled Water
dNTP	Deoxyribose Nucleoside Triphosphate
DNA	Deoxyribonucleic Acid
DNMT	Deoxyribonucleic Acid Methyltransferase
EDTA	Ethylenediaminetetraacetic Acid
ESP	Excretory Secretory Products
FBS	Foetal Bovine Serum
FISH	Fluorescence *in situ* Hybridisation

gp	Lab derived susceptible strain of *B. glabrata* (Gene Pool)
h	Hour
H3K4	Histone 3 Lysine 4
H3K4me^3	Histone 3 tri-methyl-lysine 4
H3K9	Histone 3 Lysine 9
H3K9me	Histone 3 methyl-lysine 9
H3K9me^3	Histone 3 tri-methyl-lysine 9
H3K27me^3	Histone 3 tri-methyl-lysine 27
H3K79me^3	Histone 3 tri-methyl-lysine 79
H3S10ph	Histone 3 phospho-serine 10
H4K16ac	Histone 4 acetyl-lysine 16
H4K20me^3	Histone 4 tri-methyl-lysine 20
HAT	Histone Actyltransferase
HATi	Histone Actyltransferase Inhibitor
HDAC	Histone Deactylase
HDACi	Histone Deactylase Inhibitor
HGPS	Hutchinson-Guilford Progeria Syndrome
HP1	Heterochromatin Protein 1
HS	Heat Shock
hsp70	Heat Shock Protein 70Kda
IF	Immunofluorescence
INM	Inner Nuclear Membrane
K	Lysine
KCl	Potassium Chloride
KDM	Lysine Demethylase
KMT	Lysine Methyltransferase
LAD	Lamin Associated Domain
LBR	Lamin B Receptor
LEDC	Less Economically Developed Country

LMNA	Lamin A Gene
M:A	Methanol : Acetone
M:AA	Methanol : Acetic Acid
Mb	Megabase (10^6 DNA base pairs)
MB	Methylene Blue
min	Minute
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NE	Nuclear Envelope
NM1β	Nuclear Myosin 1 Beta
NMRI	Lab derived susceptible strain of *B. glabrata* (Naval Medical Research Institute)
NPC	Nuclear Pore Complex
NTD	Neglected Tropical Disease
Nups	Nucleoporins
ONM	Outer Nuclear Membrane
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
qPCR	Quantitative Polymerase Chain Reaction
R	Remodelin
RNA	Ribonucleic Acid
RNAP	Ribonucleic Acid Polymerase
RT	Room Temperature
s	Second
S	Serine
SAM	S-adenosyl-L-methionine
SASP	Senescence Associated Secretory Product

SB	Sodium Butyrate
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean (SD/square root of the sample size)
siRNA	Small Interfering Ribonucleic Acid
SSC	Saline Sodium Citrate
Strep	Streptavidin
TDG	Thymine Deoxyribonucleic acid Glycosylase
TET	Ten Eleven Translocation
TRITC	Tetramethylrhodamine, orange fluorescent dye
UV	Ultraviolet light
v/v	Volume/volume (mL of solution/100mL)
w/v	Weight/volume (g of substance/100mL)
WHO	World Health Organisation

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Chapter 1: Introduction

1.1 – Schistosomiasis

Schistosomiasis, or bilharzia, is a neglected tropical disease (NTD), which majorly impacts endemic areas in less economically developed countries (LEDCs). Areas endemic for schistosomiasis are located in the tropics and subtropics regions including the South Americas, Africa and South Asia. The main issue caused by schistosomiasis in endemic areas is due to the effect of disability affected living years (DALY), lowering productivity and increasing required familial support to survive, that results from infection putting it on a similar level to malaria and tuberculosis for the effects it has in LEDCs (Colley *et al.*, 2014). It is a parasitic disease caused by the trematode blood flukes of the *Schistosoma* genus. The first reported case was in 1851 when German physician Theodor Bilharz described the cause of urinary schistosomiasis. Current estimates indicate that at least 230 million people may be infected in endemic areas with a further 770 million at risk of becoming infected (Walz *et al.*, 2015; Colley *et al.*, 2014; Vos *et al.*, 2012). There are three major species that make up the majority of human schistosomiasis cases. *Schistosoma haematobium* which is found in Africa and the Middle East, *Schistosoma japonicum* which is primarily found in Asia and *Schistosoma mansoni* which is found in Africa, Middle East and the South America (Colley *et al.*, 2014). There are other species that affect humans but they tend to be more localised to specific regions like *Schistosoma mekongi* which is limited to the Mekong river basin in Southeast Asia (Ohmae *et al.*, 2004). Schistosomiasis infection can generally be split into two varieties, intestinal and urinary, depending on which organ the parasites prefer to utilise for passing their eggs back into the environment. *S. mansoni* and *S. japonicum,* tend to migrate towards the blood vessels surrounding the liver and intestines whereas *S. haematobium* tend to migrate to blood vessels around the bladder.

The adult schistosomes live between 3-10 years although there are cases where they have lived for several decades within a host (Colley *et al.*, 2014). They devour the erythrocytes to gain glucose and fatty acids essential for their survival (Huang *et al.*, 2012; Barrett, 2009) which can lead to anaemia. However the majority of symptoms associated with the schistosomes are associated with the eggs they produce that are either excreted harmlessly via faeces and urine or get lodged and trapped into host tissue (Colley, and Secor, 2014; Burke *et al.*, 2009). The eggs that fail to migrate through the host's tissue into the lumen of the bowel or bladder become trapped in the tissue activating the host's immune response. This results in granuloma formation and subsequent chronic inflammation of the surrounding tissue. As an acute response this often results in the presentation of Katayama syndrome

(Burke *et al.*, 2009) generally seen in travellers or immigrants to areas where schistosomiasis is endemic who were not exposed in early childhood or *in utero*. Over time immune response to the eggs drops and the disease enters its chronic phase which differs in presentation for the intestinal and urinary forms, with a marked increase squamous cell carcinoma of the bladder being linked to chronic *S.haematobium* infection (Colley *et al.*, 2014). Ultimately all schistosomiasis infections cause disabling systemic morbidities such as anaemia, malnutrition and retarded physical development. This is more evident in endemic areas where the chronic form is rapidly achieved by constant reinfection by *Schistosoma* parasites that add the additional burden of parasite numbers to egg production. In such areas advanced indicators of disease such as hepatic fibrosis, which can take 5-15 years to develop, has been recorded in children as young as six and haematuria is viewed as part of normal development for pubescent males, similar to females' menses (Colley *et al.*, 2014). It is no surprise then that meta-analysis shows that schistosomiasis has significant long term effects on the quality of life for those in endemic areas (Vos *et al.*, 2012).

Schistosoma species have a complex life-cycle, shown in Fig 1.1, requiring both an intermediary and definitive host. *S. mansoni* as an example requires the freshwater snail *Biomphalaria glabrata* as an intermediary host and humans as a definitive host, although it can also infect hamsters, mice and other primates as definitive hosts (Blanchard, 2004). The eggs shed from the definitive host in either faeces or urine and when they come in contact with fresh water they hatch into miracidia. The miracidia swim in the fresh water and are able to survive for a couple of weeks before perishing if they do not find a suitable host. If *S. mansoni* miracidia come into contact with *B. glabrata* it will burrow into the flesh of the snail and release excretory secretory products (ESPs) (Zahoor *et al.*, 2014; Lockyer *et al.*, 2012; Zahoor *et al.*, 2009), which in susceptible snails elicit a cellular response that makes the host hospitable for the miracidia. These ESPs include *S. mansoni* variant of the metalloprotease leishmanolysin SmLeish which has been shown to affect miracidia infection and modulate immune response diminishing sporocyst encapsulation (Hambrook *et al.*, 2018). ESPs are also being investigated at differing stages of the life cycle in *S. japonicum* where ESPs from the egg can be linked to liver fibrosis formation (Gong *et al.*, 2018). In adults worms the proteome has been characterised, and established to contain multiple heat shock protein variants including HSP70, HSP90 and HSP97 which were assessed for their immunomodulation capability (Liu, F. *et al.*, 2009). HSP70 has also been shown to be important in *S. mansoni* with its inhibition affecting cercariae ability to hone in on hosts (Ishida and Jolly 2016). Once in a hospitable host the miracidia develop into primary sporocysts and to undergo an asexual reproductive process creating multiple daughter

sporocysts. The sporocysts then can produce and subsequently release multiple cercariae, the human infective stage. The development of mature cercariae in the snail can take 4-8 weeks and once they start shedding, thousands of cercariae can be shed from a snail daily whilst it survives. The released cercariae migrate in the water in search of a definitive host. If they come in contact with human skin they will burrow in and enter the bloodstream where they mature into adult schistosomes and migrate to the mesenteric vessels near the liver and intestines and where the males and females form pairs and start to produce eggs repeating the cycle.

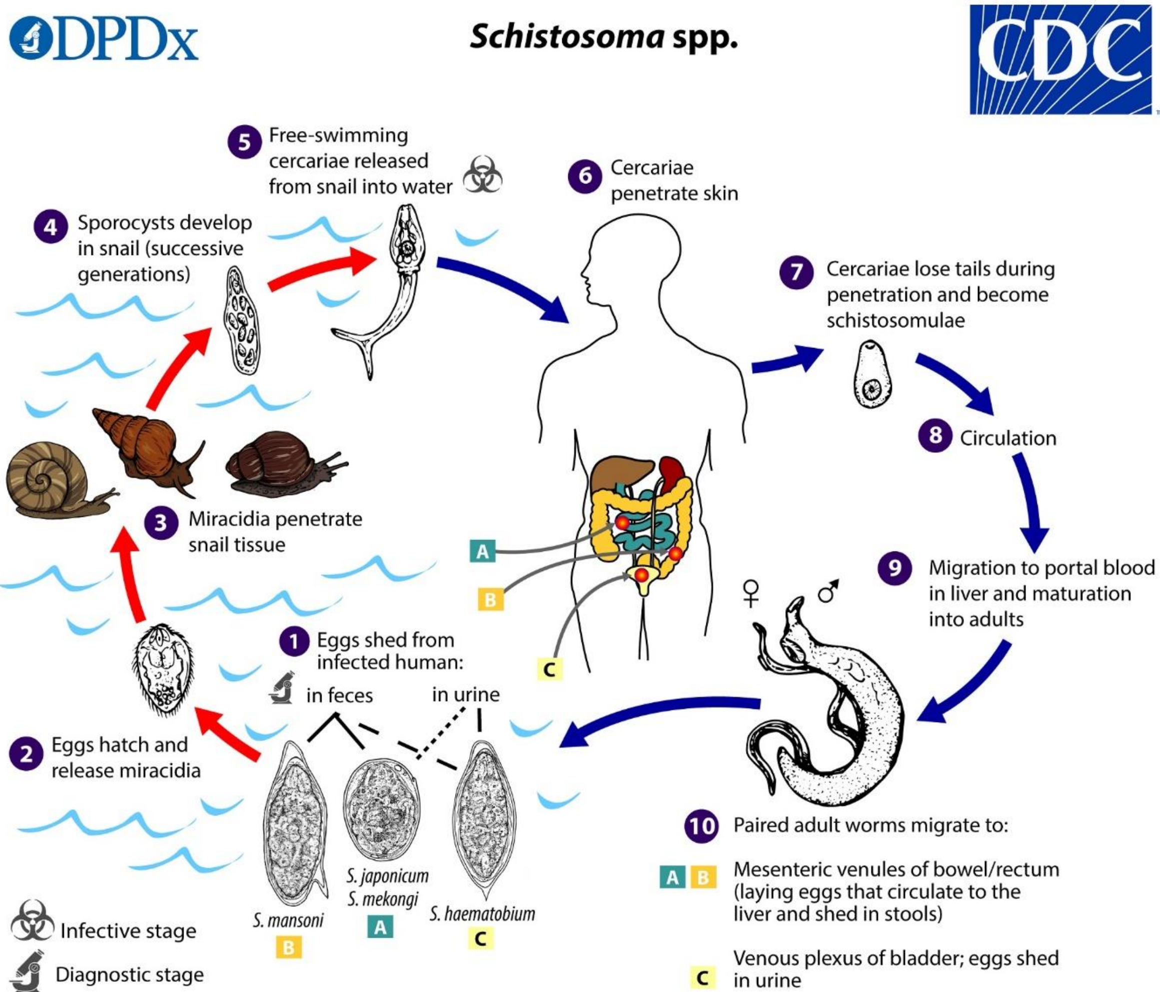


Fig 1.1 Representative diagram of the life cycle of *Schistosoma mansoni* from egg through both intermediate and definitive hosts. (Cdc.gov, 2019)

Currently to try and reduce the strain on the endemic countries, schistosomiasis is on the World Health Organisations (WHO) watch list with a statement issued about trying to

eradicate the disease in the future. Second only to malaria for its impact this is a lofty goal but the ongoing effort to do so is hampered by a lack of research into how this could be achieved. Previously efforts to control schistosomiasis spread have used molluscicides to limit *B. glabrata* populations. Although not popular now due to potential environmental impacts certain molluscicides have exhibited additional anti-cercarial effects (Augusto *et al.*, 2017). This demonstrates that molluscides could still be a viable method for control and further research into the effects of molluscicide on both the snail and parasite as well as the environmental impact of molluscides has been done (Silva *et al.*, 2018). The current WHO program for controlling schistosomiasis is that of a mass distribution of the chemotherapeutic agent praziquantel. Praziquantel is the standard treatment for infection by Schistosoma species as it is effective against all known species to infect humans (Botros *et al.*, 2005; Harder, 2002); prior to its development the drugs available were more species specific, examples being Oxamiquine and Metrifonate. The mass distribution of praziquantel once annually or biennially has been effective in controlling the spread of the disease. Yet it is a curative anti-parasitic not a preventative, it works by clearing current infection but does not offer any immunity to reinfection, it is also ineffective against juvenile schistosomes in the host (Xiao, Catto and Webster, 1985). This effectively means to be sure of clearing infection praziquantel must be taken again about six weeks after the first dose to kill off any juveniles that may have survived and are now egg producing. This of course offers no defence against reinfection by new parasites which are highly likely in endemic areas. This leaves us with two issues first is the lack of developed immunity and the second is dependency on a single drug as the bulwark to a devastating disease (Greenberg, 2013). The latter issue is one that has come up in papers since both in lab (Giboda and Smith, 1997; Fallon *et al.*, 1996) and in the field (Botros *et al.*, 2005) limited presentations of praziquantel resistance have been reported. Fortunately in both cases the resistant parasites are less viable and either die off or revert over time, yet, it opens up the distressing possibility that a resistant strain could develop.

Current research efforts aimed at the eradication of schistosomiasis is less concerned with drug development, since praziquantel seems to be standing the test of time, and on a more permanent solution which is the development of a vaccine effective against the parasite. In this regard researchers have focused both on indigenous proteins they can extract from the parasite and on recombinant proteins that have been lab developed (Bergquist *et al.*, 2002; Bergquist and Colley, 1998). The issues being faced however are twofold. First is finding proteins that are effective targets for vaccines which would allow the immune system to recognise the schistosoma parasite and the second is triggering the cellular, as well as

humoral, immune response (Redpath, Fonseca and Perona-Wright, 2014). To combat a parasite the body needs to activate both the humoral and cellular immune responses and where as we have a lot of experience with vaccines that trigger the humoral, or antibody, response it is more difficult to prime the cellular immune response which would be necessary to eradicate the parasite from the host (Oliveira *et al.*, 2008). Although no immunity has been discovered in people living in endemic areas, those who suffer recurring infection do develop a resistance over time (Oliveira *et al.*, 2008) and resistance in the intermediary host *B. glabrata* is well documented (Ittiprasert, W. and Knight, 2012; Baeza Garcia *et al.*, 2010; Coelho and Bezerra, 2006; Goodall *et al.*, 2006), if not entirely understood and maybe with a greater understanding of the processes involved in the intermediary hosts resistance to the parasite we can unlock new understandings that allow us to take a step forward to better control if not reduce the prevalence of schistosomiasis in endemic areas. Newer approaches to the development of the vaccine include DNA and synthetic peptide vaccinations (Oliveira *et al.*, 2008). DNA vaccinations seem to induce both a humoral and cellular response in the host but few have been tested in regard to a *S. mansoni* vaccination and protection offered from those that have been tested are not improved in comparison to the recombinant proteins. Synthetic peptides have the advantage of greater purity in production and potentially lower risks to the vaccinated but it is difficult selecting appropriate epitopes to replicate and several are required and need to actually be recognised by the immune system for them to be effective, which is harder to achieve since it has to take into account differences in the HLA of the target population.

1.2 – *Biomphalaria glabrata*

Biomphalaria glabrata is a fresh water snail found in warm climates and is the intermediate host for the human schistosomiasis parasite *Schistosoma mansoni*. It has become an important host model not only for schistosomiasis research but for the research community as a whole for four main reasons. First and foremost is the snail genome project that has sequenced the entirety of the *B. glabrata* genome in an effort that has taken over a decade (Adema *et al.*, 2017; Raghavan and Knight, 2006). Second is the *Biomphalaria glabrata* embryonic (Bge) cell line which is currently the only immortalised cell line available for any molluscan model, although primary cell cultures can be made they have a limited lifespan (Yoshino, Bickham and Bayne, 2013). Thirdly is the composition of the nuclei of *B. glabrata* cells which more closely resemble mammalian cells when compared to other invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Knight, M. *et al.*,

2014). Finally the *B. glabrata* model is the only model organism where it has been demonstrated that a eukaryotic pathogen is capable of manipulating the chromatin organisation of its host (Arican-Goktas *et al.*, 2014). These factors combined make *B. glabrata* an invaluable model for investigating the complex interactions of pathogenesis within a host (Bridger, Brindley and Knight, 2018).

Biomphalaria glabrata is a member of the order Mollusca gastropoda class that lives in warm fresh water preferring temperatures between 24ºC and 29ºC and although an intermediate host for *S. mansoni* the differing sub-species show well documented variations in immunity to the parasite. Adult immunity to infection is a result of Mendelian single gene dominance (Knight *et al.*, 1991), however, juvenile resistance and degree of susceptibility to infection in both juvenile and adult snails are governed by complex genetic traits (Richards, Knight and Lewis, 1992; Knight *et al.*, 1991). This variation in immunity however, has allowed investigations into how the parasite infects the host, the kind of changes that need to occur in the host organism to permit the parasite to survive. By using resistant strains such as BS-90 and susceptible strains such as BB02 and NMRI we can identify the changes the parasite elicits to improve its chances to survive by comparing what happens in the susceptible snails and what does not in the resistant snails or vice versa (Arican-Goktas *et al.*, 2014). Such changes include time dependant upregulation of *actin* and *hsp70* genes (Arican-Goktas et al., 2014). Resistance can also be altered via epigenetic changes (Knight *et al.*, 2016), as has been demonstrated with the BS-90 strain where within a generation resistance was lost simply due to changes in environmental temperature (Ittiprasert, Wannaporn and Knight, 2012).

The snail immune defence is affected by the haemolymph, more precisely it seems to be the granulocytes that consist of the hosts defence against the parasite and it is an active process (Bayne, Hahn and Bender, 2001). Initial defense reactions against a parasite, like *S. mansoni*, is to first encapsulate the invading organism (Harris, 1975). Less than half of the sporocyst's surface needs to be covered to kill the parasite (Bayne, Hahn and Bender, 2001), this encapsulation can be deterred if the *B. glabrata* homologue of a macrophage migration inhibitory factor (MIF) is knocked out or knocked down hinting at its importance for proper defence against the parasite to be elicited (Baeza Garcia *et al.*, 2010). Furthermore a unique effect protein has been discovered in the snail that is part of a family of proteins rarely found in eukaryotic organisms, biomphalysin, it is a part of the β-pore forming toxin (β-PFT) family generally found in bacteria (Galinier *et al.*, 2013). This protein is capable of

binding to the parasite and using a co-factor from the *B. glabrata* plasma cause damage to the sporocyst likely through cytolysis of its cells and caused a significant increase in sporocyst death over controls. However, the level of biomphalysin does not increase on infection by the parasite but is rather constituitively expressed indicating that it is not upregulated in response to parasite infection (Galinier *et al.*, 2013).

To study the complex interaction between host and parasite *in vitro* work using the Bge cell line can be used when studying the intermediate stages of *S. mansoni* development. The Bge cell line are cultured and immortalised embryonic cells that are adherent and show a fibroblast like morphology (Odoemelam, Edwin *et al.*, 2009; Hansen, 1976). They can be used in co-culture with *S. mansoni* to elicit the transformation of miracidia into sporocysts and ultimately cercariae allowing the mechanisms of the transformation and development to be investigated and analysed *in vitro* under controlled conditions to see what changes take place both in the host and pathogen (Coustau and Yoshino, 2000; Ivanchenko *et al.*, 1999). This also means that components of the excretory-secretory products (ESP) released by the miracidia can be isolated and tested with Bge cell populations to attempt to isolate the components that help facilitate changes in the host linked to parasite survival, these ESP products may make effective vaccination targets. However, the Bge cells can only show us so much as the cells themselves have severe aneuploidy. Normal *B .glabarata* cells have 18 chromosome pairs for a total of 36 chromosomes, the Bge cells can have as many as 67 chromosomes within a single cell (Odoemelam, Edwin *et al.*, 2009). This obviously limits the kind of experimentation that can be done with the cells alone, but, fluorescence *in situ* hybridisation (FISH) experiments on the cells themselves, targeting single copy genes *piwi* and *BgPrx4*, have shown that for the genes investigated only two copies were present (Odoemelam, Edwin *et al.*, 2009). This suggests that not all the chromosomes are replicated in the aneuploidy and experimentation on the cells may be applicable to the organism in certain cases.

The genome organisation of the *B. glabrata* nuclei is closer in structure to that seen in mammalian nuclei than that typically seen in invertebrate models (Knight, *et al* 2014) and the whole genome sequence has recently been published (Adema *et al.*, 2017). Because of this it means that *B. glabrata* is a useful model for investigating factors such as genome organisation and gene movement, especially since gene movement is shown to be a rapid process (Arican-Goktas *et al.*, 2014). In the case of *actin* it is observed that the gene was relocated from its usual position in susceptible snails within 30min but it had returned to its

usual position at the 5h time point. This gene movement also demonstrated conclusively that gene relocation, in this instance, occurred prior to upregulation of gene expression, with the increase in gene expression not measured until the 2h time point, 90 minutes after the initial movement was observed. Whereas in the resistant snails the actin gene did not have to move since it was already occupying a position similar to that which it is relocated to in susceptible snails (Arican-Goktas *et al.*, 2014). This indicates how important gene positioning and genome organisation can be for rapid response environmental stimuli and later work using a heat shock experiment which caused the *hsp70* gene to relocate mimicking the relocation observed during parasite infection. Furthermore gene relocation was halted in the heat shock model using a myosin inhibitory drug 2,3-butanedione monoxime indicating the presence of an active component, a potential nuclear motor (Arican-Goktas, 2013). Although experiments have yet to be conducted into how cessation of non-random gene relocation may affect parasite infection of its host. However, there is a complex interplay between a host and pathogen interactions as has been previously demonstrated even within the same population of snails and parasites (Galinier *et al.*, 2017) as such inhibiting a single method of action, such as gene relocation may not, on its own, be enough to afford resistance to infection.

1.3 – Nuclear Organisation

The nucleus is a complex organelle responsible not only for the protection of the genetic material from damage but for the organisation and provision of essential machinery for gene transcription. The nucleus itself is made up of a complex collection of structures and nuclear bodies with distinct purposes. The nuclear envelope (NE) which separates the chromatin from the cytoplasm, is a complex interaction of two membranes, the outer nuclear membrane (ONM) and the inner nuclear membrane (INM) that are separated from one another by about 50nm (Hetzer, 2010) and supported from within by the nuclear lamina. The NE is perforated by nuclear structures called nuclear pores, which sit in specialised wells where the ONM and INM meet to create the pore membrane (Wente and Rout, 2010). The pores core structure is made up of eight proteins which creates a cylindrical opening that is 30nm in diameter and 50nm in length to traverse the lumen between the ONM and INM. It is through these pores that transport into and from the nucleoplasm occurs, the function of the pore is contingent on the interactions its binding sites have with other proteins. Nucleoporins (Nups) associate with the nuclear pore complex (NPC) and set up the permeability membrane due to containing a high percentage of phenylalanine-glycine

repeats and mediates active transportation through the NE (Hetzer, 2010). It is these permanently bound Nups and other proteins that bind either at certain points in the cell life cycle or in a cycle of attachment and removal from the NPC that determine what macromolecules can be transported through the nuclear pore (Wente and Rout, 2010).

Both the ONM and INM have proteins associated with them unrelated to the NPC. ONM localised proteins are associated with nuclear position and possibly with the passing of signalling from cytoplasm to the nucleoplasm via interactions between the KASH domain of the ONM localised proteins and the Sad1p/UNC-84 domain of INM localised proteins (Hetzer, 2010). INM localised proteins include the lamin B receptor (LBR), lamina-associated polypeptides and emerin which interact with the nuclear lamina and potentially involved in spatial positioning and anchorage of chromatin within the nucleoplasm (Demmerle, Koch and Holaska, 2013; Boyle *et al.*, 2001). The INM localised proteins often associate with the nuclear lamina, a membrane within the nuclear envelope that is made up of the lamin proteins that fall into two subsets of A-type and B-type and it is this membrane that connects the nuclear envelope to the chromatin (Bridger, J. M. *et al.*, 2007). The lamins are of particular interest as a range of diseases have been linked to mutations with the proteins, now called laminopathies, which includes Hutchinson-Guilford progeria syndrome (HGPS) where mutation in the LMNA gene which results in a truncated lamin A protein leads to the appearance of rapid ageing and on the cellular level chromatin dysregulation (Gonzalez-Suarez, Redwood and Gonzalo, 2009). The lamina offers support to the bilayer nuclear envelope while at the same time interacting with and helping maintain the stability of the chromatin contained within the nucleus and has an effect on histone markers associated with epigenetic silencing of chromatin (Camozzi *et al.*, 2014).

Another greater structure to the nucleus is the nuclear matrix (NM) sometimes referred to as the nuceloskeleton and likened to the similar cytoskeleton found in the greater cell body. This is a thin filamentous structure made up of protein and RNA that lends form to nucleus and is believed to remain event through cell division as a starting point for reconstitution of the nucleus (Elcock and Bridger, 2010). The NM has been described several times and is essentially the left over structure when all the soluble material from the nucleus has been extracted. The NM can bind to and tether the telomeric regions of the chromatin, interact with the lamina proteins and one way that chromatin organisation may be controlled is through this interaction but it also provides a platform onto which other nuclear bodies can be anchored such as transcription factories (Elcock and Bridger, 2010).

Contained within the nucleus are several nuclear bodies that seem to have transcriptional capabilities but are distinctly different. The most commonly researched of these are the transcriptional factories which are situated on the NM of the nucleus and range in size from 40-100nm, the active component within being a RNA polymerase (RNAP), with RNAP II being the one most commonly found in the nucleus (Elcock and Bridger, 2010). Splicing speckles are a dynamic nuclear body that contains a reservoir of inactive RNAP II and an abundance of splicing machinery (Xie, S. Q. *et al.*, 2006). However, these bodies have been shown to move around and associate with NM, at any point there can be 20-40 separate speckles contained within a single nucleus and they are generally found near actively transcribing genes (Zhang, Qiao *et al.*, 2016). PML bodies are another nuclear structure that are associated with the NM, the extent of its functions are not known but, the best observed is the recruitment of proteins for SUMOylation to occur and there is evidence that PML bodies are dispensable as $pml^{-/-}$ mice have shown no obvious biological defect (Lallemand-Breitenbach and de Thé, 2010). Then there are Cajal bodies and histone locus bodies, two different nuclear bodies often found in near proximity to, or associated with, one another. The signature of the Cajal body is the coilin protein found within the small structure and its responsibility for processing of snRNA, histone locus bodies are mainly responsible for processing of the pre-mRNA for histone proteins, they also often have high levels of coilin but the functional relationship between the Cajal and histone locus body is not known (Nizami, Deryusheva and Gall, 2010).

When you combine these factors it is hardly surprising that the chromatin contained within the nucleus is not randomly distributed, but rather each chromosome occupies specific chromosome territories within the interphase nucleus (Cremer, T. and Cremer, 2001). In general organisation within the nucleus has heterochromatic gene poor regions closer to the nuclear periphery and euchromatic gene rich regions towards the nuclear interior, as demonstrated in Fig 1.2, although the inverse can be found in certain cells such as the rod cells of the eye in nocturnal mammals (Solovei *et al.*, 2009). Non-random locationing of chromosomal territories has also been demonstrated in 3D (Schmälter *et al.*, 2014; Bolzer *et al.*, 2005). The specific territories that a chromosome will occupy are not absolute as entire chromosome territories can move given the correct stimuli, such as forcing a cell into quiescence or senescence (Dillinger, Straub and Németh, 2017; Rutledge *et al.*, 2015; Bridger, J. M., Boyle, Kill and Bickmore, 2000). Similarly single genes can be seen to move during cell differentiation (Szczerbal, I., Foster and Bridger, 2009) or be forced to do so by

infection (Knight, *et al.*, 2011a). Gene movements can also be highly specific, gene relocation is known to occur in cancer transformation but where those genes relocate can be tissue specific (Meaburn *et al.*, 2016). The specific spatial orientation of chromosomes within the nucleus has been shown to affect when DNA is replicated (Heinz *et al.*, 2018) and single gene relocations have been associated with changes in transcription (Arican-Goktas *et al.*, 2014). Some of these genes can be shown to be moving on their own without the entirety of the chromosome following and when they do loop out on chromatin fibres to associate with specific nuclear structures or bodies (Szczerbal, Izabela and Bridger, 2010). How this movement occurs is a major point of interest as they can move over significant distances in a relatively short space of time giving credence to the idea that there are nuclear motors at work within the nucleus (Bridger, J. M., 2011).

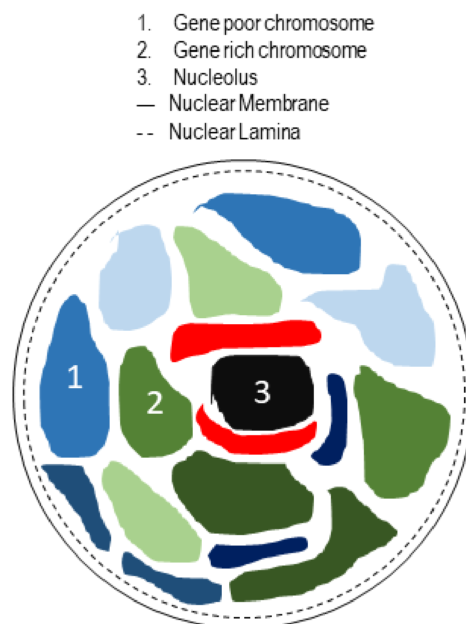


Fig 1.2 Representative image of chromosome territory domains within the nucleus. 1) Gene poor chromosomes located at the periphery. 2) Gene rich chromosomes located within the nuclear interior. 3) Nucleolus of the nuclei, outer solid ring is the nuclear membrane and the inner perforated ring the nuclear lamina.

Furthermore several factors have been identified within chromatin that potentially affect spatial organisation. Lamin associated domains (LADs) which are areas of chromatin closely associated with the nuclear lamina and the interplay between the LADs and the lamina can affect peripheral positioning of chromatin (Forsberg *et al.*, 2019). Topologically

associating domains that produce the diverse folding of chromatin within the chromatin domains (Fudenberg *et al.*, 2016). Although it has recently been demonstrated that these TADs are somewhat fluid and malleable within a single cell population (Finn *et al.*, 2019). Other factors that govern spatial organisation may include DNA replication machinery and DNA repair proteins as has been implicated by high-throughput imaging mapping (Shachar *et al.*, 2015). However, changes that signal for the genes to relocate or for differentiation to occur are not well understood and these extra-genetic signals that affect gene profile and genome organisation and are collectively referred to as epigenetics.

Although the nucleus is highly organised, changes in organisation can occur for several reasons including age, disease and environmental stimuli. These changes are occasionally dynamic and quick to resolve as during infection (Arican-Goktas *et al.*, 2014) others like changes during senescence become permanent (Bridger *et al.*, 2000). What is necessary for these movements to occur is a mechanism by which chromatin can be moved. Within the cytoplasm movement is facilitated by actin-myosin motors (Wollrab *et al.*, 2019) and there is evidence that the same motor function may assist in *Plasmodium* locomotion (Kumar *et al.*, 2019). Both of these proteins are found within the nucleus as well, actin forms part of the aforementioned nucleoskeleton (Xie, X. and Percipalle, 2018) and nuclear myosins have been isolated (Nowak *et al.*, 1997). One hypothesis for rapid active chromatin rearrangement involves a nuclear motor made of these two proteins similar to those found in cytoplasm and this has been supported by nuclear actin and myosin inhibition experiments preventing chromosome relocation (Mehta, I. S. *et al.*, 2010). This hypothesis is further supported by the fact that a nuclear myosin inhibitor has been demonstrated to inhibit *hsp70* non-random gene loci relocation (Arican-Goktas, 2013).

1.4 – Epigenetics Overview

The term epigenetics was coined in 1942 by Conrad Waddington, currently it refers to the study of inheritable elements that survive mitosis and meiosis but does not affect the underlying genetic structure. This broad statement currently encompasses at least four areas of separate but interlinked research. These areas are DNA methylation, histone modification, non-coding RNA (ncRNA) and finally chromatin conformation (Zhang, Guoqiang and Pradhan, 2014). All these can and do effect the expressions of genes and maintained through both meiosis and mitosis. However, the epigenetics of a cell can be

altered by many factors making it difficult to grasp the true impact of such a dynamically changing system.

The most widely studied epigenetic modification is the DNA methylation pattern. DNA is typically modified at CpG islands, which are stretches of DNA at least 200bp in length that have a higher than would be expected number of cytosine and guanine residues. In the mammalian genome 30% of CpG islands are found within the transcriptional start site of genes and 32% are found within the main body of the gene itself. The majority of CpG islands are stable either methylated or not and will remain that way however approximately 21.8% of the CpG islands in the human genome are dynamically methylated switching between states (Ziller *et al.*, 2013). DNA methylation has three potential methods of action that is by altering either the transcription factor binding affinity, the chromatin conformation or recruiting methylation specific recognition factors to promotors or gene bodies thereby repressing the expression of that gene.

DNA methylation is governed by the DNA methyltransferases (DNMT), the DNA methylation patterns are relatively stable and maintained through multiple cell divisions and potentially inheritable via germline cells. Because of this, DNA methylation is seen as the archetypal epigenetic marker. Initially, within the mammalian genome DNA methylation is caused by DNMT3a and DNMT3b during embryonic development, however later control and continuation is governed by DNMT1 (Li, E. and Zhang, 2014). However, despite this continuity provided by DNMT1-mediated copying of methylation states to daughter cell the DNA methylation state is not permanent with the pattern changing due to several factors including cellular ageing and environmental challenges. DNMT1 will methylate DNA at unmethylated CpG islands however, it preferentially targets hemimethylated sites where one strand of DNA has been methylated but the other is not, as with the DNA in the daughter cells after mitosis, showing preferential maintenance of existing patterning over establishing new methylation marks. This kind of activity was postulated in 1975 (Holliday and Pugh, 1975). Yet this does not account for how methylation marks can be changed.

There are two ways whereby this could occur, passively or actively. The passive route is by continual dilution, the DNMT family proteins responsible for CpG methylation get blocked and as the cell passes through the cell cycle each successive daughter cell will have a gradual reduction in methylated CpG. Active reduction of DNA methylation has several

proposed methods of action including but not limited to base excision repair (BER) mediated by Gadd45 and 5-methylcytosine (5mC) deamination by AID/APOBEC with subsequent mismatch repair (Zhang, Guoqiang and Pradhan, 2014). However the latest proposed method is via the action of Ten Eleven Translocation (TET) dioxygenases that would have 5mC oxidised to 5-hydromethylcytosine (5hmC) which is in of itself an emerging epigenetic marker. From 5hmC it would be converted to 5-formylcytosine (5fC) and then 5-carboxylcytosine (5caC) which can be removed by thymine DNA glycosylase (TDG) and repaired by BER (Li, E. and Zhang, 2014).

Maintaining the correct DNA methylation profile is essential for maintaining the health of the cell. The loss of DNA methylation in a cell leads to aberrant transcription occurring and incorrect DNA methylation patterns have been linked to disease such as cancer (Meldi and Figueroa, 2015), auto-immune (Lei *et al.*, 2009), neurodegeneration and neurodevelopment defects. 5hmC itself is a key component for neural health and development in murine models (Sun, W. *et al.*, 2014) so the TET model of active 5mC removal by hydroxylation means alteration or loss of 5mC patterning is going to affect 5hmC patterning which could account for the neuronal effects in both cases (Santiago *et al.*, 2014).

NcRNA refers to sections of DNA that were once considered Junk DNA because they do not encode for a protein, now they can be referred to as dark regions, sections of DNA that we have yet to discover a purpose for. ncRNA are RNA elements that affect the transcription and or translation of DNA to protein and several varieties of ncRNA have been identified (Zhang, Guoqiang and Pradhan, 2014). Long non-coding RNA (lncRNA) is one of the most important, these RNA are more diversely expressed than protein encoding genes and play roles in cell differentiation, organ development, chromatin modification and even in X chromosome silencing with the Xist lncRNA being involved in directing both the DNA methylation of the silenced X chromosome but also its hypoacetylation (Liu and Pan, 2015; Nie *et al.*, 2012). Small interfering RNA (siRNA) and micro RNA (miRNA) both help in controlling translation of RNA into protein by attaching to transcribed RNA and preventing it from being translated into protein by indicating it for destruction (Carthew and Sontheimer, 2009). A newer and less understood ncRNA is the piwi-interacting RNA (piRNA) which form complexes with PIWI and are proposed to silence transposable elements of the DNA and protect overall genomic integrity (Hirakata and Siomi, 2016).

Histone modifications are another well researched mechanism of epigenetic variation. Histones are proteins that assist in DNA condensation by forming an octamer made up of histone proteins, two of each H2A, H2B, H3 and H4, these octamers are referred to as a nucleosome to which DNA is attached and wrapped around twice (Luger *et al.*, 1997). Each nucleosome binds to approximately 146 base pairs of DNA (Luger *et al.*, 1997). The nucleosomes themselves have varying affinities for both the DNA and other proteins affecting how condensed the resulting chromatin is causing the differentiation we see between euchromatin and heterochromatin. These differences in protein affinity and chromatin condensation are driven by alterations in the histones that make up the octamer caused by post translational modifications to the component histones. These post translational modifications can take several forms and some are mutually exclusive generally occurring on the N-terminal tail of the histone proteins although it can occur within the globular region of the histone as well (Zhang, Guoqiang and Pradhan, 2014). The most researched and commonly found alterations to histones are methylation, acetylation and phosphorylation. Decoding what has been referred to as the histone code had been a focus of research for decades and yet still more modifications are being found and what has been investigated shows a complex interplay between different histone modifications and other epigenetic markers such as DNA methylation (Hervouet *et al.*, 2018).

Finally there is the conformation of the chromatin itself. Chromatin in the interphase nucleus occupies specific three dimensional territories of the nucleus. The way the chromatin folds and forms itself into that three-dimensional structure is going to affect how accessible certain genes and promoter regions are (Cremer, M. *et al.*, 2017; Kieffer-Kwon *et al.*, 2013), how close long range enhancer elements are to the site they effect and formation and interaction of gene domains with transcriptional mechanisms such as RNA polymerase II (RNAP II) transcription factories (Rowley *et al.*, 2019), Cajal bodies (Wang, Q. *et al.*, 2016), splicing speckles (Chen, W. *et al.*, 2018) and similar. This chromatin conformational folding and looping can be captured using chromosome conformation capture technology (3C) but part of this folding is going to be influenced by the currently expressing epigenetic markers such as histone modifications and DNA methylation. In fact there is extensive cross-talk becoming apparent between the various epigenetic modifications with CpG island methylation binding complexes such as MeCP2 having active histone deactylases and/or histone methylation sites indicating that methylation of certain CpG island methylations have an effect on the neighbouring histone modifications (Nagano *et al.*, 2013). Histone modifications can recruit DNMT family proteins affecting the DNA methylation of associated DNA and several ncRNAs have been discovered that target DNA methyltransferase

essentially indicating extensive cross interactions between the epigenetics at several disparate levels that affects overall gene expression of the cell without affecting the contents of the genetic information contained within it.

1.5 – Histone Modifications: Acetylation

Histones are integral proteins involved in the condensation and organisation of DNA into chromatin within the nucleus, histones H2A, H2B, H3 and H4 form the nucleosome core around which DNA wraps (Luger *et al.*, 1997) with HP1 (heterochromatin protein 1) variants assisting in greater binding and condensation (Hiragami-Hamada *et al.*, 2011) as well as DNA damage recognition (Soria and Almouzni, 2013). The histone proteins that make up the nucleosome are subject to dozens of post translational modifications that alters their interaction with the DNA bound to them and with neighbouring nucleosomes changing the accessibility of the DNA to transcriptional mechanisms within the nucleus as well as the potential stability of the genetic code (Jenuwein and Allis, 2001). Currently several mechanisms have been identified to modify the histone proteins the most commonly researched are histone methylation and acetylation (Zhang, Guoqiang and Pradhan, 2014). However, phosphorylation, ubiquitination, sumoylation, biotinylation and several other mechanisms have been identified, generally altering amino acids found in the NH_2-terminal tail of the histone proteins (Zhang, Guoqiang and Pradhan, 2014) although some modifications have been found within the main globular structure of the protein (Ng *et al.*, 2002). The most commonly affected amino acid are the lysine (K) residues that can be methylated, acetylated, ubiquitinated and sumolyated, although arginine (R), histidine (H), serine (S), threonine (T) and tyrosine (Y) residues have also been identified as targetable residues. Histone modifications tend towards a more dynamic existence with known mechanisms for adding and removal of acetyl, methyl and phosphoryl groups from histones and several pathways of histone modification have been explored for specific gene activations in model systems (Zippo *et al.*, 2009) and in the general aspect (Konsoula and Barile, 2012; Suganuma and Workman, 2008) so there is an understanding of what certain modifications generally indicate.

The two most well researched histone modifications are histone acetylation and methylation. Histone acetylation is handled by histone acetyltransferases (HATs) and histone deacetylases (HDACs) which are responsible for the addition or removal of the acetyl group

(Grunstein, 1997). Histone acetylation is associated with transcriptionally active genes. The mechanism for this association is unclear but it is currently theorised that since acetylation occurs on the epsilon-amino group of conserved lysine residues that the acetyl groups negative charge antagonises the DNA phosphate backbones negative charge resulting in weaker binding of the DNA to the histone causing the chromatin to unravel slightly allowing transcriptional mechanisms to access the DNA (Zhang, Guoqiang and Pradhan, 2014). The most obvious example of such an unravelling would be the acetylation H4K16 which in of itself inhibits chromatin fibre folding for a 30nm section of DNA (Shogren-Knaak *et al.*, 2006). H4K16ac is in fact a part of a series of histone modifications and protein recruitments that can activate gene transcription (Zippo *et al.*, 2009). The same modification is also involved in the dosage compensation seen in *D.melanogaster* for upregulation of specific genes on the X chromosome in males to counterbalance only having a single gene copy (Gelbart *et al.*, 2009).

The first nuclear HAT was discovered in *Tetrahymena* and subsequent investigations started to indicate similar sequences to that of the HAT active site in other proteins previously identified, namely Gcn5 in yeast (Brownell *et al.*, 1996) and the mammalian GCN5. Gcn5 was already identified as a transcriptional regulator within yeast cells showing a close link between histone acetylation and gene transcription. In fact many of the HATs that have been identified are subunits in larger nuclear complexes responsible for transcriptional activation (Roth, Denu and Allis, 2001). However, the HATs have also shown to be extremely well targeted with each HAT only acetylating a few specific lysine residues (Roth, Denu and Allis, 2001). A key example of this specificity is MOF which is responsible for the hyperacetylation of H4K16 of the male *D.melanogaster* X chromosome (Gelbart *et al.*, 2009). Similarly MOF is involved in H4K16ac in mammalian cells as part of gene transcription activation (Zippo *et al.*, 2009).

HATs have been divided structurally into two differing families, Gcn5-related *N*-acetyltransferase (GNAT) (Vetting *et al.*, 2005) and MYST, named after the first four identified members (Roth, Denu and Allis, 2001). Both the GNAT and MYST families share a common motif which contains an Arg/Gln-X-X-Gly-X-Gly/Ala amino acid sequence and it is this motif that is important for interacting with the acetyl coenzyme A (acetyl-CoA) from which the HATs get the acetyl group to transfer to the histones (Roth, Denu and Allis, 2001). Although both families share this motif GNAT all share at least three similar motifs to one another (Vetting *et al.*, 2005; Roth, Denu and Allis, 2001) whereas the acetyl-CoA motif is

the only one MYST family members have but many MYST family proteins also contain zinc fingers and chromodomains that are for protein to protein interactions often found in heterochromatin-associated proteins (Carrozza *et al.*, 2003; Roth, Denu and Allis, 2001). GCN5 obviously belongs to the GNAT family as does HAT1, MYST family includes MOF and TAT-interactive protein with a 60KDa mass (Tip60) which was the first identified human MYST family member. Interestingly hMOZ and MORE are also MYST family HATs but contain plant homeo domains (PHD) (Roth, Denu and Allis, 2001) which can bind to methyl-lysine on histones (Pedersen and Helin, 2010), showing a potential crosstalk between histone acetylation and methylation. Initially p300/CBP, which refers to two HATs which are relatively interchangeable, were althought to belong to their own small family however, closer examination of the motifs contain within the protein demonstrated that the protein shared three motifs in common with the GNAT family (Roth, Denu and Allis, 2001).

HDACs like the HATs were first isolated in the 90's, a team investigating methods to return transformed spindle-like NIH3T3 cells back into normal fibroblasts found that treating the transformed NIH3T3 with trapoxin caused the transformation to reverse. When further experimentation were done on the trapoxin treated NIH3T3 cells it was discovered they had hyperacetylated histones (Kijima *et al.*, 1993). It was in discovering the target for trapoxin that they were able to identify the first HDAC (Taunton, Hassig and Schreiber, 1996). At present eighteen HDACs have been identified in humans and they are split into four separate classes, class I HDACs 1, 2, 3 and 8, class IIa HDACs 4, 5, 7 and 9, class IIb HDACs 6 and 10, class III sirtuins (SIRT) 1-7, and class IV HDAC 11 (Bertrand, 2010). Class I, II and IV HDACs are all zinc dependant in their action, whereas class III are nicotinamide adenine dinucleotide (NAD) dependant, and related to acetylpolyamine amidohydrolases (APAHs) and acetoin utilisation proteins (Leipe and Landsman, 1997). This is evident, not due to sequence similarity but, due to crystal structure determination of the proteins. Arginase contains three loops L3, L4 and L7 that coordinate with two manganese ($Mn^{2+)}$ ions where as HDACs have two conserved loops L4 and L7 coordinated with a single zinc (Zn^{2+}) ion, this same α/β fold is observed in HDLP, HDAC-like amidohydrolase (HDAH) and APAH showing a conserved evolution from a common metalloprotein ancestor (Lombardi *et al.*, 2011). Interestingly HDAC classes I, II and IV are all susceptible to inhibition by trichostatin A (TSA) whereas the sirtuins are not inhibited (Bertrand, 2010).

Class I HDACs are so categorised due to their similarity to the Rpd3 yeast gene and are generally localised to the nucleus of the cell and ubiquitously expressed in all tissues (Thiagalingam *et al.*, 2003). HDAC1 and HDAC3 can deacetylate all four histone proteins that make up the nucleosome at every normally targeted lysine residue however they have differing affinities for certain targets suggesting that, although they can globally deacetylate the histones if need be, there is targeting of specific lysine residues. The most researched HDAC is HDAC8 which is made up of 377 amino acids a little smaller than the average of 400-500 for most class I and lays on the boundary between classes I and II and has been mapped to the Xq13 by fluorescence in situ hybridisation (FISH) (Van den Wyngaert *et al.*, 2000). Inhibition of HDAC8 results in hyperacetylation of histones 3 and 4 showing a more targeted approach. However HDAC8 activity is controlled by phosphorylation of the protein by cyclic AMP-dependant protein kinase A (PKA) which causes HDAC8 activity to decrease (Wolfson, Pitcairn and Fierke, 2013; Somoza *et al.*, 2004). Conversely there is experimental data showing that phosphorylation of HDAC1 and HDAC2 is correlated to increase in activity (Segré and Chiocca, 2010). HDAC1 is also a target for SUMO-1 (small ubiquitin-related modifier) which may also potentially regulate HDAC1 activity (David, Neptune and DePinho, 2002), it should also be noted that when class I HDACs are inhibited by TSA there is a related upregulation of messenger RNA (mRNA) for HDAC1-3 but not for HDAC8 hinting at an autoregulatory loop with negative feedback increases production of HDACs by transcriptional machinery that HDAC8 is not a part of (Thiagalingam *et al.*, 2003). It is also becoming more evident that HDACs like HATs tend to be part of larger gene transcription regulatory complexes with HDAC1 and HDAC2 associating with Sin3 complex and CoREST and HDAC3 with silencing mediator for retinoid and thyroid hormone receptor complex (SMRT) and nuclear receptor corepressor (N-CoR) (Cress and Seto, 2000).

Class II HDACs were discovered once Hda1 a yeast deacetylase was identified and several human homologues were isolated, HDAC4-7 and HDAC9-10, these HDACs are approximately 1000 amino acid residues in length making them much larger than the class I HDACs (Bertrand, 2010; Thiagalingam *et al.*, 2003). Class II gets further subdivided due to their nature, all class II HDACs have a secondary catalytic domain within their structure, class IIa have them in the COOH terminus region where as class IIb has it in the NH_2 terminus region. HDAC10 although it has a NH_2 terminus catalytic domain does have a pseudorepeat similar to a catalytic domain in the COOH terminus as well (Thiagalingam *et al.*, 2003). This class of HDACs differs from the class I in the fact that they are both cytoplasmic and nuclear in origin and can pass through the nuclear envelope and are more tissue specific. They also generally form parts of larger multiprotein complexes, in some

cases the same complexes as the class I HDACs and there is definite associations been made between HDAC4/5 and HDAC3 with the N-CoR complex (Fischle *et al.*, 2002).

Class III are a family of proteins that bear similarity to the yeast Sir2, called sirtuins seven have been identified in humans SIRT1-7 (Bertrand, 2010). These rely on a NAD-dependant activity that catalyses the acetyl moiety of acetyllysine. Only SIRT1, SIRT2 and SIRT7 have defined histone targets, with SIRT2 targeting H4K16 deacetylation that would allow condensation of the 30nm segment of chromatin that is prevented from folding by the H4K16ac form (Inoue *et al.*, 2007). This may be connected to the role of SIRT2 with cell cycle control prior to entering M-phase. SIRT1 is also linked to several functions involved in metabolism, cell cycle and lifespan alongside the actions in epigenetic variation by targeting several specific histone acetylations (Inoue *et al.*, 2007) yet, it requires interaction with HDAC4 for increased stability, although for SUMOylation rather than deacetylation (Han *et al.*, 2016).

Class IV only contains a single protein, HDAC11, at a size of 347 residues it is closer in size to class I (Gao *et al.*, 2002). However, it is not ubiquitously expressed and is limited to kidney, heart, brain, skeletal muscle and testes. This limited expression is more in line with class II HDACs and the HDAC11 catalytic site, which takes up the majority of the sequence, has homologous similarity to both class I and II (Gao *et al.*, 2002). It is the smallest of the HDACs, located primarily in the nucleus and forms a complex with HDAC6 showing that like most HDACs there is interaction between themselves and with other proteins (Gao *et al.*, 2002). HDAC11 is of particular interest due to its upregulation in certain cancers such as breast and colon and the fact that inhibition of HDAC11 in such cases can result in tumour cell apoptosis (Deubzer *et al.*, 2013). Several of the HDACs have evolved to support multiple functions and have other target substrates rather than just histones, an example of this is HDAC11's diverse role in immune cell differentiation (Yanginlar and Logie, 2018; Yuan *et al.*, 2018). They also have a complex interplay amongst themselves which is what allows them to deal with the diverse and ever changing nature of the epigenetics of a cell.

1.6 – Histone Modifications: Methylation

Histone methylation is another well-known modification that can occur on epsilon amino group of a lysine and has been identified since 1960s however, the first histone lysine methyltransferase (KMT) was not identified and isolated until nearly four decades later in 2000, SUV39H1 (KMT1A) (Black, Van Rechem and Whetstine, 2012). SUV39H1 is the human homolog of the *D.melanogaster* Su(var)3-9 gene so called due to translocation experiments done with *D.melanogaster* that showed postion-effect variegation (PEV) where euchromatic genes when juxtaposed next to heterochromatic regions would become silenced. Su(var) (suppressor of PEV) refers to the fact that the genes transposed next to it would suffer from suppression of expression resulting in a variegation in gene expression, one example being the suppression of the *white* gene in *D.melanogastor* which is normally expressed throughout the eye resulting in red colouration only being partially expressed ending with a speckled red and white colouration (Grewal and Elgin, 2002; Muller, 1930). Histone lysine modifications occur on specific lysine residues within the histone protein but also in specific states that subtly alter the effect, lysine residues can be mono-, di- or tri-methylated (Yun *et al.*, 2011; Cheung and Lau, 2005) and are generally associated with one of three particular effects, formation of heterochromatin, gene activation involving transcription and elongation via RNA polymerase II or silencing of genes in euchromatic regions (Zhang, Guoqiang and Pradhan, 2014).

SUV39H1 became the prototypical KMT and as such was dissected for the functional site that would signify the lysine methyltransferase activity. The enzymatic SET domain was identified and had been highly conserved, from the Su(var)3-9 *D.melanogaster* protein, was 130 amino acid residues in length (Dillon *et al.*, 2005). Using this other KMTs were able to identified, SUV39H2, G9a and SETDB1 to name a few. The SET domain uses S-adenosyl-L-methionine (SAM) as the donor for the methyl group that shall be added to the lysine residue. However, a second class of KMT has been identified, which currently only has one member, that does not have the SET domain, this is KMT4 (Dot1L) (Zhang, Wen and Shi, 2012; Nguyen and Zhang, 2011), which uses the same SAM substrate but with a unique N-terminal section with the C-terminal sharing similarity with other SAM-dependant methyltransferases. The crystalline structures of the SET-domain KMTs is notably different from other SAM-dependant methyltransferases arranging the SAM and epsilon amino group of the lysine at opposites of the catalytic site with a narrow tunnel for the substrate to enter by but, this places it directly next to the methyl group being transferred from the SAM and

keeps the SAM as far from the phosphate backbone of the DNA wrapped around the histone as possible (Dillon *et al.*, 2005; Zhang *et al.*, 2002). This arrangement is believed to have developed to allow progressive methylation of substrates without disassociation from the protein.

KMTs have shown a remarkable specificity not only for the target substrates but also for the methylation states that they are responsible for, KMT1A for instance targets mono-methylated H3K9 (H3K9me) and will further methylate it to either a di-methylated or tri-methylated state (Black, Van Rechem and Whetstine, 2012). This specificity can be altered as was demonstrated with mutation studies of KMT7 which normally mono-methylates H3K4 can be altered to a tri-methylation or di-methylation if a mutation occurs at Y245A (Xiao. *et al.*, 2003) or Y305F (Zhang *et al.*, 2003) respectively. Similarly mutation of F281Y mutation alters DIM-5, a yeast SET-domain containing KMT, from a tri-methylase to a mono- or di-methylase. This particular mutation has become known as the F/Y switch which can establish the SET-domain substrate specification (Dillon *et al.*, 2005).

HATs and HDACs were isolated relatively close to one another; however, the first KDM was not isolated until 2004 (Shi *et al.*, 2004). Up until this point it was believed that histone methylation, like DNA methylation, was a long term epigenetically inheritable marker that was removed by the action of proteolysis and replaced with new histone proteins as necessary (Jenuwein and Allis, 2001). While it is true that histone methylation is more enduring and does not appear to be altered as rapidly, with the average half-life of a methylation mark being approximately equal to that of the histone itself (Cheung and Lau, 2005), two distinct classes of protein have been identified that have been shown to possess histone demethylase activity. The KDM1 family which uses flavin adenine dinucleotide (FAD)-dependant amine oxidase domain and the Jumonji C (JmjC) domain containing protein, which are either Fe^{2+} or α-ketoglutarate dependant (Pedersen and Helin, 2010).

KDM1A/AOF2/LSD1 was the first histone lysine demethylase discovered, it was also shown to be highly selective in terms of substrate only demethylating mono- (me1) and di-methylated (me2) H3K4 but did not tri-methylated (me3) H3K4 (Shi *et al.*, 2004). KDM1A has since proven to have significant roles in embryo development, cell differentiation (Wang. *et al.*, 2007) and cell proliferation of neuronal cells (Sun, G. *et al.*, 2010). KDM1A has also been a focus in cancer research, although this requires further dissecting as in some tumour

cell lines it can be prognostic of poorer outcomes (Lim *et al.*, 2010) and in other instances its expression may be linked to beneficial actions (Wang. *et al.*, 2009). The KDM1 family of proteins comprises the only two amine oxidase domain containing KDMs, the second being KDM1B. Both target the H3K4me1/me2 for demethylation with similar structures containing a SWIRM domain and amine oxidase catalytic domain (Burg *et al.*, 2015) however, they have inherently different targets. KDM1A targets the promoter regions of genes (Adamo *et al.*, 2011) and is embryonically lethal in knockout mice models by the eighteenth day of embryogenesis (Wang *et al.*, 2007). Whereas KDM1B knockout is not embryonically lethal but, results in infertility in females as DNA methylation patterning can't be established in oogenesis (Ciccone *et al.*, 2009) and the KDM1B targets methylations found in the gene body rather than the promoter (Fang *et al.*, 2010). As with the KMTs small changes can alter KDM1B activity, it has two binding sites required for recognition of the substrate and removing the second, although it does not completely prevent demethylation of H3K4me2 does result in only 50% of the target substrates being demethylated with the other 50% being reduced to H3K4me1 instead of being completely demethylated (Chen *et al.*, 2013).

KDM2A was the first demethylase enzyme to be discovered that did not have an amine oxidase catalytic domain and instead relied on a JmjC domain. This enzyme used a Fe^{2+} and α-ketoglutarate as co-factors in the reaction to demethylate H3K36me1/me2 variants (Tsukada, *et al.*, 2006). This discovery also opened up the research of KDMs as the JmjC domain containing protein family was significantly larger than that of the amine oxidase catalytic domain containing family of proteins (Tsukada, *et al.*, 2006). The KDM2 family has a diverse role in the maintenance of genome stability with KDM2B being linked to protection from UV- induced apoptosis and oxidative stress (Polytarchou *et al.*, 2008; Koyama-Nasu, David and Tanese, 2007). It is also capable of overcoming the senescence barrier for cells which may link to some proto-tumorigenesis effects (Pfau *et al.*, 2007) and yet this capability is also important for natural embryonic development (He *et al.*, 2008).

Other KDM subfamilies include the KDM3 subfamily of proteins contains three identified mammalian enzymes, KDM3A, KDM3B and JMJD1C however only KDM3A and KDM3B are have been shown to have histone demethylase activity with JMJD1C variants unable to do so (Brauchle *et al.*, 2013). KDM3A and KDM3B both target the same substrates, H3K9me1/me2 and minor changes, such as a T667A substitution of amino acids can drastically affect enzymatic activity (Brauchle *et al.*, 2013). The KDM4 subfamily of proteins was the first to show target specific against tri-methylated lysine residues. There are five

proteins contained within this family comprising KDM4A-E, KDM4A-D all have target specificity to H3K9me2/me3 with all but KDM4D also targeting H3K36me2/me3 (Zhang, Wen and Shi, 2012). KDM4A-C are globally present in tissue, although A and C are expressed in greater amounts than B, while D and E are predominantly found in the testes (Labbé, Holowatyj and Yang, 2013). Functional tests involving the KDM4 family has shown that KDM4A-C preferentially target H3K9 residues for demethylation over H3K36, with all five preferentially targeting tri-methylated over di-methylated residues (Hillringhaus *et al.*, 2011). The KDM5 subfamily consists of four proteins in mammalian models KDM5A-D all of which have the same substrate specificity, H3K4me2/me3 (Horton *et al.*, 2016). Another family that can demethylate tri-methyl marks from histones their roles in the body seem to be rather diverse and potentially tissue specific. KDM5A when it was knocked out in a mouse model showed only minor changes in phenotype (Klose *et al.*, 2007). Whereas KDM5B is down regulated in adult tissue but, found to be upregulated in several cancer cell lines and its inhibition can slow tumour growth (Horton *et al.*, 2016). However, like KDM3A, it is important in the maintenance and self-renewal of ES cells (Xie *et al.*, 2011) and it is also an important component in DNA double strand break repairs (Li *et al.*, 2014). KDM5C is also of note since it seems to have significant effects on neuronal development as mutations in *KDM5C* gene can be linked to mental retardation and potential autism spectral disorder (Adegbola *et al.*, 2008; Tzschach *et al.*, 2006; Jensen *et al.*, 2004).

Finally The KDM6 subfamily is made up of three proteins, KDM6A-C, and are responsible for demethylating H3K27me2/me3. KDM6A, also called UTX, has been shown to be important in regulation of HOX genes in *C.elegans* and other models (Agger *et al.*, 2007; Lan *et al.*, 2007). Although KDM6B does also mediate HOX gene regulation it is also activated during infection within macrophages and seems to be an important factor within T-helper 17 (Th17) cell differentiation (Liu *et al.*, 2015; De Santa *et al.*, 2007). Both KDM6A and KDM6B have been shown to be important for final differentiation of T-cells in general (Manna *et al.*, 2015), yet more recent work is casting doubt on the role these two play in development on mammals as a recent study has shown that even without KDM6A or KDM6B murine embryonic cells can demethylate H3K27me3 and activate their HOX genes (Shpargel *et al.*, 2014). Similarly the KDM6C gene, also called UTY, is the Y chromosome variant of UTX which was althought to be incapable of demethylase activity has shown that unlike in the murine model (Shpargel *et al.*, 2012) the Human variant of the gene does in fact maintain demethylase activity for the same substrate although is not as effective as the KDM6A/UTX (Walport *et al.*, 2014).

1.7 The Ageing Nucleus

Ageing is the gradual decline of function experienced over time of organ systems and tissue experienced by all living organisms, although some organisms that are able to reverse this state becoming pseudo-immortal like *Turritopsis dohrnii* (Lisenkova *et al.*, 2017) and *Turritopsis nutricula* (Devarapalli *et al.*, 2014) jellyfish which are a focus in research into ageing there is only so much that can be learned from these models. Ageing is a multifaceted natural occurrence and benefits from study in multiple organisms at different levels. Several models have been used over the years including *D. melanogaster*, *C. elegans* and *Mus musculus*. Due to work in these models calorie restriction has been identified as one of the possible, non-genetic, interventions for improving lifespan and the processes it effects are being further investigated (López-Lluch and Navas, 2016). However, progeroid syndromes, like Hutchinson-Guilford progeria syndrome (HGPS) which resembles premature ageing in humans are an indication of just how important the nucleus is to the ageing process. Progeria cells develop abnormalities in nuclear morphology (Bridger and Kill, 2004) over time that impact on nuclear and genome organisation and reversal of this phenotypic change has been used to identify potential treatments (Bikkul *et al.*, 2018; Scaffidi and Misteli, 2005) as a way of preventing premature senescence in affected cells. Although some recent work in fish has shown that nuclear morphology changes and premature ageing may be independent of one another (Tonoyama *et al.*, 2018). This does indicate however, that there is a potential link between nuclear health and senescence.

The accumulation of senescent cells is theorised to be one of the events that contribute to the phenomena of ageing. Senescent cells are cells that are no longer capable of proliferating leading to degradation of tissue. It has been shown that nuclear organisation is affected when cells leave a proliferating state to enter a senescent state (Bridger *et al.,* 2000) and organisational changes can be driven by proteins such as high mobility group A proteins (Narita *et al.*, 2006) or by the accumulation of structures such as nuclear pores, although specific components such as the nucleoporin TPR may ultimately be responsible for nuclear organisation changes (Boumendil *et al.*, 2019). These senescent cells also effect normal proliferating cells around them, encouraging them to become prematurely senescent (Nelson, G. *et al.*, 2012). It has also been demonstrated that targeted elimination of senescent cells positively impacts on lifespan and health (Baker *et al.*, 2016). However, changes to senescent cells are not limited to genome organisation patterns but, also

changes in epigenetics. Changes to DNA methylation patterns have been linked with age related alterations in lifespan (Cole *et al.*, 2017), in fact such changes in DNA methylation are rigorous enough to estimate age physical age and models have been develop for human (Hannum *et al.*, 2013; Horvath, 2013) and murine (Meer *et al.*, 2018) models.

Similarly other epigenetic markers have been investigated for connections to ageing. One marker that has been associated with age is the H3K27me^3 marker. H3K27me^3 marks are gradually accumulated within the genome as an organism ages (Ma *et al.*, 2018; Hosogane *et al.*, 2016). Alterations to H3K27me^3 patterning could also be linked to senescence with the formation of cytoplasmic chromatin fragments (CCFs) which are transcriptionally repressed fragments of chromatin extruded from the nucleus in its own vesicle (Dou *et al.*, 2017). These CCFs have been shown to mediate senescence associate secretory products (SASPs) which are in part responsible for inflammatory effect of senescent cells and potentially for the bystander effect to surrounding cells. Either way alterations to the nuclear health and integrity ultimately impacts not only on the individual cell but the surrounding microenvironment. Ageing therefore works as an additional variable for exploration of the host-pathogen interaction, how these factors that affect chromatin distribution, protein expression and surrounding microenvironment affects the parasites ability to manipulate its host may further elucidate the mechanisms involved in the host-pathogen interaction.

1.8 Summary

In summary *B. glabrata* as a model allows for diverse investigations into multiple topics. With its similarity to mammalian nuclear organisation and shared parasitic disease, schistosomiasis, the complex interplay between host and pathogen interactions can be explored. The current hypothesis would indicate that the ESPs from the miracidia of *S.mansoni* can cause rapid global alterations to genome organisation within its host *B. glabrata*, potential mechanism is demonstrated in Fig 1.3, and one of the most likely ways to affect this change is via epigenetic alterations. There is evidence that the gene relocation noted in infection occurs prior to gene upregulation which would suggest the purpose for gene movement if gene activation. The epigenetic targets for such changes would be histone acetylation, histone methylation of specific targets or DNA demethylation or DNA 5-hydromethylcytosine. Since histones and their respective modifications are well conserved

initial investigation into a link between parasite-induced gene movement will focus on alterations to the histone code alongside inhibition of histone modifying proteins.

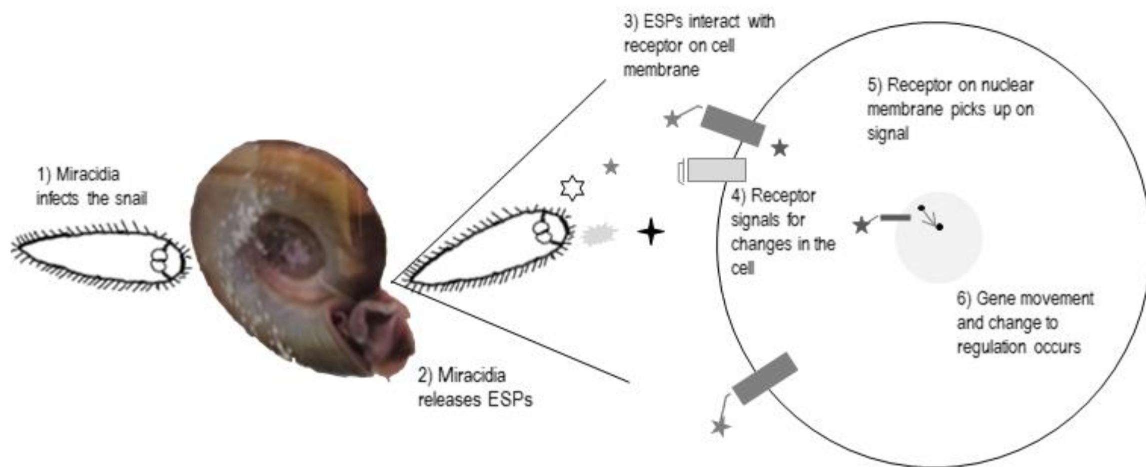


Fig 1.3 Representation of possible mode of action for the miracidia to induce gene relocations via excretory secretory products (ESPs). 1) Miracidia infects snail. 2) Releases ESPs. 3) ESPs interact with cell receptors. 4) Cell receptor signals for changes in the cell releasing a nuclear signal. 5) Nuclear receptor receives signal from cell receptor. 6) Nuclear receptor induces changes to genome organisation resulting in gene relocation.

Furthermore combining this with the fact that *B. glabrata* is a comparatively short lived model organism the effects of age can be explored in relation to both infection and genome organisation. Finally inducible gene movement has already been established within the *B. glabrata* model both from parasite infection and heat-shock and in both occur pretty rapidly, within 15mins in the case of infection. As such there needs to be mechanisms that drive non-random gene relocation and since this occurs rapidly during infection the most likely modifications to elicit these effects are changes to epigenetic markers and motor proteins would need to be present in the nucleus to effect the signalled for movement. As such the relationship between infection or heat shock, epigenetics and age can be explored focusing on the effects these have on genome organisation and rapid gene relocation.

Chapter 2: Investigating epigenetic alterations associated with stress factors known to induce genome reorganisation in the molluscan model *Biomphalaria glabrata*

2.1 Introduction

The field of epigenetics is a large and ever expanding area of study incorporating chromatin positioning, non-coding RNA, DNA methylation and histone modification. Histone modifications have an established role in maintaining gene expression with several well categorised modifications being linked to either gene activation such as H3K4me^3 (Chen *et al.*, 2015; Kim *et al.*, 2009), H4K16ac (Gelbart *et al.*, 2009) and H3S10ph (Zippo *et al.*, 2009; Suganuma and Workman, 2008), or inactivation such as H3K27me^3 (Pan *et al.*, 2018; Wiles and Selker, 2017; Pushpavalli *et al.*, 2012) and many work in concert for further modulation of gene activity. These modifications have also been found to alter chromatin compaction as is the case with H4K16ac (Shogren-Knaak *et al.*, 2006), which could be one mechanism by which increased gene activation is achieved by allowing easier access to the DNA for transcription.

Similarly, it is well established that the nucleus is a highly organised organelle (Fritz *et al.*, 2019; Szczepińska, Rusek and Plewczynski, 2019; Fritz *et al.*, 2015; Boyle *et al.*, 2001; Croft *et al.*, 1999) with chromosomes occupying specific chromosome territories. However, during certain events such as differentiation (Szczerbal, Foster and Bridger, 2009; Kim, S. H. *et al.*, 2004) and infection (Knight, *et al.*, 2011a; Li, C. *et al.*, 2009) chromosome and gene movements occur. In the case of *Schistosoma mansoni* infection of *Biomphalaria glabrata* there is clear evidence of movement prior to upregulation of gene expression (Arican-Goktas *et al.*, 2014). While with adipogenesis in porcine mesenchymal cells gene movement can be seen to be directed to splicing speckles (Szczerbal and Bridger, 2010) which would indicate directed movement of genes within nuclei. Considering the relative overlap in what is known one possible indicator for movement within the genome could be alterations to the histone code which could then be read by nuclear motors to indicate where the gene or chromosome needs to be moved to.

Two techniques commonly used for investigating nuclear organisation are immunofluorescence (IF) and fluorescence *in situ* hybridisation (FISH) for visualising and localising proteins and gene/chromosome positioning respectively. Generally the use of the two methods together requires optimisation of 3D FISH due to differences between IF and 2D FISH fixations. 2D FISH uses an acidic fixation to flatten the nuclei into a 2D conformation, this acidic fixation can cause denaturation of certain proteins making

optimisation of combined 2D FISH and IF complicated and on an “as case basis”, one commonly used 2D FISH-IF application that has been shown to work for Ki67 staining of proliferating cells. Here are explored methods to improve the efficacy of a combined 2D FISH-IF approach by optimising IF for histone proteins in an acidified fixative.

One of the genes induced by *S. mansoni* infection of *B. glabrata* is *hsp70* (Arican-Goktas *et al.*, 2014) which is a well-known inducible gene within many species (Hu *et al.*, 2009; Boehm *et al.*, 2003). The HSP70 family of proteins are in both the cytoplasm and nucleus but and protect the cell by binding to and preventing the aggregation of misfolded or heat damaged proteins (Lindquist and Craig, 1988). It was found to be inducible after heat shock of BB02 strain *B. glabrata* snails in a manner similar to the gene movement seen during infection. Investigations were undertaken to assess whether significant alterations were made to the histone modification distribution throughout nuclei following heat shock which could be indicative of changes to genome organisation or gene expression in response to the environmental stress. It is anticipated that changes to the epigenome, such as changes to the histone code, are necessary for altering genome organisation and therefore influences gene positioning. As such four histone modifications were selected, all of which were methylation markers. Two markers for activation and two for inactivation were selected, the active markers were H3K4me^3 (Chen *et al.*, 2015; Kim *et al.*, 2009) and H3K79me^3 (Nguyen and Zhang, 2011). H3K79me^3 was of particular interest as RNA sequencing data had indicated it was affected during *S. mansoni* infection making it a potentially important target for parasite/infection induced gene relocation. The first inactivation marker chosen was H3K27me^3 (Wiles and Selker, 2017) H3K27me^3 is involved in several processes including ageing (Ma *et al.*, 2018; Dou *et al.*, 2017) and *B. glabrata* susceptibility is known to change as they age (Richards and Minchella, 1987). H4K20me^3 is a second inactivation marker which is associated with chromatin compaction and is related to a transition from proliferation to quiescence (Evertts *et al.*, 2013), affected during some viral infections (Teferi *et al.*, 2017) and shown to be associated with the innate inflammatory response (Stender *et al.*, 2012). Demonstrating that H4K20me^3 is a marker that is liable to change during infection if the parasite infection includes immunomodulation of the host.

Thus this chapter investigates optimising the existing 2D FISH fixation method for use with IF for histone antibodies in *B. glabrata*. Characterises the nuclear patterning for four histone modifications using indirect IF on adult *B. glabrata* in comparison to stress response to establish if global alterations to histone modification distribution can be ascertained that

could indicate association with gene repositioning. Following establishment of a conical pattern distribution in the adult snail three modifications, $H3K27me^3$, $H3K79me^3$ and $H4K20me^3$ were investigated in juvenile snails post heat shock and 2h post infection to assess if changes to histone distribution due to either environmental stress (heat shock) or biological stress (*S. mansoni* infection) mimicked each other.

ChIP has become a standard technique for investigating protein DNA interactions (Collas, 2010). Yet this is mainly used for global high-throughput techniques such as ChIP-seq. This is because optimisation for a targeted ChIP-qPCR experiment is fraught with issues such as, ChIP buffer optimisation, antibody suitability and target sites for primers. ChIP buffers that vary from protocol to protocol can cause issues such as that seen with $H3K79me^3$ histone modification experiments in human T-cells, where despite the $H3K79me^3$ marker being linked to transcriptionally active genes in yeast, it had a modest correlation with transcriptionally silenced genes in human cells (Barski *et al.*, 2007). Later it was found that using a buffer which contained SDS revealed specific epitopes for H3K79 methylation which proved that $H3K79me^3$ was indeed associated with transcriptionally active genes (Steger *et al.*, 2008). Similarly commercially available antibodies have reliability issues even from batch to batch (Taussig, Fonseca and Trimmer, 2018; Voskuil, 2014) and although highly conserved there is no guarantee that antibodies designed to work in one organism will function, especially when targeting a new model organism such as *Biomphalaria glabrata*, or that a certified ChIP ready antibody will actually work during a ChIP protocol. Finally, a further issue is where on the gene the primers should be targeted since there is no knowledge of where the histone modifications may be within the chromatin sequence or if they are even there to begin with.

To facilitate the development of more targeted investigations of gene – histone modification interactions a method is required to bridge the gap between theorised presence of a histone modification and ChIP to allow more accurate use of ChIP-qPCR. By combining IF and FISH it would be theoretically possible to identify if histone modifications are associated with a gene prior to ChIP. However, the two procedures require different fixations if performed in 2D and with 3D it may be difficult to distinguish where exactly the associations are within the gene even if the association was clear through all the other histone modification signals from unrelated chromatin containing the marker. A method by which gene signal and histone modification association can be made unambiguously is required. Proposed here is the use of immuno-fibre-FISH, a method for examining single chromatin fibres for both gene signal

and histone modification markers which would be visible within the gene signal itself, this would act as a validating step prior to ChIP-qPCR and potentially used for gathering quantative data should optimising ChIP prove difficult.

2.1.1 Aims

- To assess if changes to histone modification pattern distribution occur after stress factors known to induce chromatin remodelling
- To optimise Methanol : Acetic Acid (M:AA) fixation to work with histone antibodies for IF
- Development and optimisation if fibre-FISH protocol

2.2 Methods

2.2.1 Snail Husbandry

The BB02 strain of *Biomphalaria glabrata,* a wild type strain susceptible to *Schistosoma mansoni* infection originally isolated from Brazil, was kept in a dedicated snail room with a recirculation system consisting of multiple 6.5L tanks where the water temperature was maintained at 27°C. The system was mains fed and the water is filtered through a series of sediment filters, carbon filter and reverse osmosis membrane and then essential salts are re-added to the water. A 12 hour dark/light cycle was maintained and the snails fed with fish flake. Faeces, debris and excess egg masses were removed from the tanks every two days. Maintaining this environment meant that snails reached maturity in 6 – 8 weeks and egg masses were laid at a rate of 2 per day/snail.

2.2.2 Cell Suspensions from *Biomphalaria glabrata* tissue for Indirect Immunofluorescence

Suspensions for indirect immunofluorescence (IF) were prepared using the ovotestis of two snails. The shells were crushed using a microscope slide and needle nose tweezers were used to remove the shell and extract the ovotestes, Either the tweezers or a scalpel were used to excise the tissue which was then placed into a sterilised 1.5mL microcentrifuge tube

containing 0.5mL of 1X PBS. Using a tissue grinder the tissue was macerated until no large tissue lumps were visible in the solution. A further 0.5mL of 1X PBS was added, mixed by inversion and the suspension left for 30min at room temperature (RT). Following this incubation the samples were spun at 200g for 5min and the supernatant discarded. Samples were then washed once by resuspending in 1mL 1X PBS and centrifuged at 200g for 5min, this step was sometimes repeated depending on the amount of debris, until the supernatants were clear. After discarding the supernatant the cells were fixed in 1mL of 4% paraformaldehyde (PFA) (Fisher Scientific, UK) in 1X PBS, the cells were resuspended in the fixative and incubated at RT for 10min. Samples were then centrifuged at 200g for 5min, supernatant discarded and the pellet resuspended in 1mL 1% Triton X-100 (Sigma Aldrich, UK) in 1X PBS and incubated at RT for 10min to permeabalise the cells. The suspension was then spun at 200g for 5min supernatant discarded and resuspended in 1mL 1X PBS, spun again at 200g for 5min and finally resuspended in 0.5mL 1X PBS and stored at 4°C for up to two weeks.

2.2.3 Environmental Stressing of *Biomphalaria glabrata*

Snails were subjected to one of two environmental stressors, heat shock (HS) and cold shock (CS). For HS a water bath was set to 32°C and a beaker filled with sterile water was placed in the water bath to be brought up to temperature. Once at the desired temperature snails were placed into the beaker and left for 2h. For CS a polystyrene box was filled with water, a beaker with sterile water added to the box and ice added around the beaker creating an ice bath. Temperature in the beaker was carefully monitored and once the water reached 16°C snails were added to the beaker and the temperature monitored to ensure it did not fluctuate for 2h. Snails subjected to the environmental stressor were then dissected as per section 2.2.2.

2.2.4 Parasite infection of *Biomphalaria glabrata*

Snails were transferred from Brunel University London to the Wellcome Sanger Institute two days prior to infection to allow them to acclimatise and prevent stress from the journey negatively affecting the experiment. Infections were performed on an individual basis, this was achieved by using a 24-well plate, each well had 10 miracidia added to it and then 1mL of lepple aquarium water added. Snails were then placed into individual wells for 2h. All parasite work was performed in temperature controlled environment keeping the snails at the

preferred 28ºC to avoid adding additional stressors. Once infection was completed snails were dissected as described in section 2.2.2.

2.2.5 Indirect Immunofluorescence

100µL of snail cell suspension (2.2.2) was pipetted onto the top of a poly-L-lysine coated slide propped at 45º to encouraging coating of the entire slide with enough cells for later analysis and prevent clumping in a single region. Slides were then placed on a slide dryer set at 37ºC until the solution had evaporated and cells had fully adhered to the slides. They were then washed in 1X PBS for 5min while gently shaking to removed dried salts, excess liquid was then drained off onto paper towel. The primary antibody was diluted in 2% FCS in 1X PBS and 100µL was added to each slide, see table 2.1 for optimised dilutions of antibodies. The slide was then covered with parafilm and placed into a humidified chamber overnight at 4ºC. Slides were then washed in 1X PBS for 15min on a shaker with three changes of buffer. The secondary antibody was diluted in 2% FCS in 1X PBS and 100µL was then pipetted onto the slide and covered with parafilm before being placed in a humidified chamber at 37ºC for 30min. Slides were then washed in 1X PBS for 5min on a shaker three times, rinsed off in sterile dH_2O and excess liquid was drained onto tissue paper. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) [1.5µg/mL] (Vectashield anti-fade mountant, Vector Laboratories). Slides were visualised and images taken using a fluorescence microscope (Olympus BX41 fluorescence microscope). Distinct patterns seen within the nuclei from the histone modifications were then distinguished and scored for 1000 nuclei in triplicate. Number of nuclei scored, in triplicate from 3 biological replicates, was done so due to the mixed nature of the cell pool taken from the ovotestes and would minimise the effects any single cell type might have on scoring. Samples scored comprised one of four categories, unstressed (controls), heat shocked, cold shocked or 2h post infection by *S. mansoni*. The variations in pattern seen between control and stressed snails were compared using a Two-Tailed, Equal Variances Student T test.

Antibody	Dilution Factor
H3K4me^3	1:200
H3K27me^3	1:200
H3K79me^3	1:400
H4K20me^3	1:200
Donkey Anti-Rabbit TRITC	1:200
Goat Anti-Rabbit Alexa Flour 488	1:500

Table 2.1: Displays the optimised dilution factors for the primary and secondary antibodies used for indirect immunofluorescence.

2.2.6 Optimisation of Methanol : Acetic Acid fixation for Indirect Immunofluorescence

Cell suspensions were prepared from ovotestes, excision of which is outlined in 2.2.2, and placed in 0.5mL of 0.05M KCl solution and macerated using an tissue grinder (Axygen Scientific, UK) and a further 0.5mL of 0.05M KCl added to the samples, mixed by inversion and left to incubate at RT for 30min prior to centrifugation at 200g for 5min. Supernatants were discarded and methanol : acetic acid (3:1) fix added one drop at a time followed by agitation of the sample until it was at the correct cellular density and left to incubate at RT for 10mins. Samples were then centrifuged at 200g for 5min, supernatant discarded and fixative added dropwise with agitation and incubated at RT for 10min, fixation step was repeated at least three times. Samples could be stored at -20°C but fixative was changed each time they were removed from storage.

Cell suspensions were dropped from height onto wet slides to get the appropriate cell spread. Excess liquid was drained off the slides dried on a slide warmer set to 37°C. Optimisation was 30min at 37°C, room temperature for 1-2h or 4°C overnight (primary) or 4h (secondary) permeations for both primary and secondary antibodies. Overnight primary incubation and secondary incubation at 37°C for 30mins proved to be most effective. Further tests were performed using ageing of the slides once dropped and RNase A washes to clean slides and reduce debris and finally the addition of permeablisation steps were tried to reduce background including use of 0.5%-5% Triton X-100 (Sigma Aldrich, UK), 1% octylphenoxy poly(ethyleneoxy)ethanol, branched (IGEPAL CA-630 NP40) or NP40 (Sigma Aldrich, UK), 1% Tween 20 (Sigma Aldrich, UK) and 1% Saponin (Sigma Aldrich, UK).

2.2.7 Comparison of Methanol : Acetone Fixation and 4% Paraformaldehyde Fixation

Methanol : Acetone (M:A) fixation was performed in the same manner as described in section 2.2.6 with a M:A at a 1:1 ratio replacing the methanol : acetic acid (M:AA) fixation and 1X PBS replacing the 0.05M KCl. M:A fixed cells can be stored at -20ºC indefinitely. M:A slides can be dropped as described in section 2.2.6 and 4% PFA slides were prepared as described in 2.2.5. Both fixation methods were compared to one another for overall antibody penetration of cells, background fluorescence and potential patterning. These were then compared to M:AA fixed cells to assess if any changes occurred to patterning due to the acidic fixation.

2.2.8 Bge Cell Culture

Bge cells were grown in 25cm^2 cell culture flasks using media consisting of 22% Schneider's Drosophila medium, 0.45% (w/v) lactalbumin hydrolysate, 0.13% (w/v) galactose, 10% charcoal stripped FBS (Sigma Aldrich, UK) which has been previously heat inactivated at 56ºC with gentamicin at a final concentration of 50µg/mL. Phenol Red was added to the medium to a concentration of 14.1µM to give indication when the pH of the medium starts to drop and should be changed. Cells were split once a week using a sharp tapping and cell scraping to dislodge the cells. The cell suspension was transferred to 15mL falcon tubes and spun at 1000rpm for 5mins the supernatant was then aspirated from the pellet. Flasks were reseeded at a 1:6 dilution by re-suspending cell pellet in 3mL and adding 0.5mL of the resuspension to 9.5mL of media in the flask. Cell culture flasks were maintained at 27ºC in sealed flasks, no atmospheric alterations necessary.

2.2.9 Harvesting Genomic DNA from Bge Cells

Bge cell suspension was spun at 400g for 5mins, the cell medium removed and the cells washed in 3mL 1X PBS before being spun at 400g for 5mins and the 1X PBS removed. Once spun the cells were re-suspended in 1mL of 1X PBS and transferred to a 1.5mL micro-centrifuge tube and spun at 400g for 5mins and the supernatant discarded and the cells re-suspended in 400µL of digestion buffer consisting of 100mM Tris [pH 8.0], 200mM sodium chloride, 5mM EDTA and 0.2% SDS (w/v). Once re-suspended 40µL of proteinase K [20mg/mL] was added and incubated at 55ºC for at least 2h. After digestion the mixture was vortexed and then centrifuged at 17000g at 4ºC. The supernatant was transferred to a fresh

tube and DNA precipitated by adding 10% 3M sodium acetate (v/v) and 2x volume ice cold ethanol (v/v) and incubated the mixture at -20ºC until DNA was visible. DNA was transferred using a Pasteur pipette to a fresh tube containing 70% ethanol, washed and then spun at 170g for 5mins and supernatant discarded and replaced with fresh 70% ethanol. This was repeated once more and then spun and the supernatant discarded before air drying and re-suspending the DNA pellet in 200µL of ddH_2O.

2.2.10 Labelling of BAC probes and preparation for Fluorescence *in situ* Hybridisation

Probe labelling was done using a nick translation kit (BioNick™, Invitrogen, UK) to incorporate Biotin-14-dATP and to cut the probe into 200-500bp in length. Combined in a 0.2mL micro-centrifuge tube was 5µL 10X dNTP mix, 1µg DNA and made up to 45µL with distilled water and 5µL 10X enzyme mix added to a final volume of 50µL. The contents were mixed then centrifuged for 5s at 10000g at which point it was incubated at 16ºC for 50min. The probe was run on a 2% agarose gel to ensure that probe size was between 200-500bp. Unincorporated nucleotides were removed using Microspin G50 columns (GE Healthcare, UK) and the probe collected in a new 1.5mL micro-centrifuge tube.

Prepping the probe for FISH requires 250ng of probe DNA, 40µg Bge genomic DNA and 3µg herring sperm DNA for each slide. The DNAs are mixed together and 10% total volume of 3M sodium acetate (v/v) and 2.25x volume of ice cold 100% ethanol (v/v) was added. This mixture was incubated at -80ºC for 30min or -20ºC for at least 1h then centrifuged at 400g at 4ºC for 30mins. The supernatant was discarded and the pellet washed with ice-cold 70% ethanol and centrifuged at 400g at 4ºC for 15mins. The supernatant is then discarded and the probe dried in a hot-block at 56ºC and subsequently dissolved in 12µL hybridisation mix (50% formamide (v/v), 10% dextran sulphate (v/v) and 1% Tween 20 (v/v) in 2X SSC) per slide. Prior to use the probe was denatured using the hot-block by adding distilled water to a well and a temperature of 75ºC, once the well has reached temperature the micro-centrifuge tube with the probe was placed in the well for 5mins. After denaturing the probe is incubated at 37ºC for at least 10mins before use.

2.2.11 Cell Suspensions from *Biomphalaria glabrata* tissue for Fibre-FISH

Cells were excised from *B. glabrata* as described in 2.2.2. Once ovotestes were removed they were placed in 0.5mL of 1X PBS in a sterile 1.5mL microcentrifuge tube and macerated using a tissue grinder until no large tissue clumps remain. The sample was then spun at

400g for 5min and the supernatant discarded and 1mL of 1X PBS added and the pellet re-suspended.

2.2.12 Preparation of Chromatin Fibres on slides for Fibre FISH

10µL of the live cell suspension from 3.2.4 was diluted in 90µL of 1X PBS and the cells counted using a haemocytometer. Once the total cells are known the original solution was vigorously agitated and diluted in 0.05M potassium chloride (KCl) at a cell density of $1x10^5$ – $4x10^5$ and left to incubate at RT for 15-30min. Using a Shandon Cytospin 2 Centrifuge 500µL of the cell suspension were spun onto poly-L-lysine coated slides. Slides were placed vertically in Coplin jars containing a lysis solution (2.5mM Tris-HCl pH7.5, 0.2M NaCl, 1% Triton X-100 (v/v) and 2M Urea, modified from Prof Beth Sullivan's protocol (Sullivan, 2010)). Slides were incubated at RT for 30min in lysis buffer and then slowly removed from the solution and a clean microscope slide was then scrapped down along the slide to drag out the chromatin fibres from the softened nuclei. Immediately after this the slide was placed in a Coplin jar containing 4% PFA for 10min at RT and transferred to a Coplin jar containing 0.1% Triton X-100 (v/v) in 1X PBS and incubated for 10min.

2.2.13 IF on Chromatin Fibres

Following preparation of the chromatin fibres as described in 3.2.5 the blocking buffer was prepared, 0.01% Triton X-100 (v/v) and 0.5% BSA (w/v) in 1X PBS. Slides were placed into a Coplin jar containing the blocking buffer and incubated at RT for 15-30min. Primary antibody, $H3K79me^3$ (abcam, UK), was diluted 1:400 in blocking buffer. After blocking slides were removed and excess blocking buffer drained off using a paper towel and then 25µL of the primary antibody is added to the area containing the fibres. Fibres were covered with a piece of parafilm and placed in a humidified chamber and incubated at 4ºC for 24-48h. Slides were then washed three times in Coplin jars containing 0.05% Tween 20 (v/v) in 1X PBS (PBST) at RT. Parafilm was allowed to float off in the wash buffer and the Coplin jar was not agitated during the wash, this preserved the fibres that can be easily scratched off. Slides were transferred from one Coplin jar to the next during washing. The secondary antibody was diluted in blocking buffer, 1:200 for donkey anti-rabbit TRITC (Jackson Labs) and 1:500 for goat anti-rabbit Alexa Fluor 488 (ThermoFisher UK). After the final wash excess liquid was drained off and 25µL of the secondary antibody was added to the slide where the fibres are contained and covered with parafilm before being placed in a humidified chamber and incubated at RT for 1h. Secondary was washed off using three washes in

PBST following the same protocol as for the primary antibody washes. One slide was taken, rinsed in sterile dH_2O, drained the excess liquid off onto tissue paper and then counterstained with DAPI [1.5µg/mL] (Vectashield anti-fade mountant, Vector Laboratories). This was used to ensure staining of the fibres had worked and fibres were still in good condition prior to continuing on with the protocol with the remaining slides.

2.2.14 FISH on Fibres

Following preparation of fibres as per 3.2.5 protocol DNA was denatured by placing slides in 70% Formamide in 2X SSC pH 7.0 at 72°C for 90s. 22x22 coverslips were prepared on a heated surface with 10-12µL of BAC probe prepared as described in section 3.2.3. After denaturing the DNA slides were immediately removed from the solution and excess liquid dried off with tissue paper while ensuring the area with the fibres did not dry out. The areas containing the fibres were then brought to the probe and bubbles under the coverslip eliminated before sealing the coverslip using rubber cement. Slides were then left for 24-72h at 37°C in a humidified chamber to hybridise.

2.2.15 Post Hybridisation Washes

After hybridisation the rubber cement was removed from the coverslips. Slides were washed in 2X SSC at 42°C for 5mins three times. After which slides were blocked using the blocking buffer in 3.2.6 for 15-30min at room temperature in a covered Coplin jar. A Strepavidin-Cy3 conjugate was diluted in blocking buffer at a 1:200 dilution. After blocking the excess blocking buffer was removed and 25µL of the diluted antibody added to the fibres and covered with a square of parafilm placed in a humidified chamber and left at 4°C for 24-48h. The antibody was washed off using 2X SSC for 5min, 1X PBS with 0.1% Tween 20 for 1min and finally 1X PBS for 1min. After the final wash excess liquid was drained off the slide and fibres were counterstained with DAPI [1.5µg/mL] and covered with a glass 32x22 cover slip. Slides were then visualised and images captured using an Olympus BX41 fluorescence microscope, UPlanFLN 100x/1.30 oil immersion objective and model viewpoint GS gray scale digital camera (Digital Scientific) or Leica DM4000, Leica PL Fluotar 100x/1.30 oil using a Leica DFC365 FX camera.

2.2.16 IF and FISH on Fibres

After 2.2.13 but prior to 2.2.14 IF antibodies are fixed in place by using 4% PFA for 10mins after which slides are stored in PBST until ready for hybridisation. Two separate protocols were used to try and maintain fibre integrity. Protocol A as outlined in 2.2.14 and Protocol B as outlined here. Protocol B used the Top Brite automated FISH system. Probes were prepared as per 2.2.10. As the probes reannealed with the blocking gDNA 22x22 glass coverslips were prepared and placed on a heated surface and the Top Brite prepared setting it to hybridise for 2min at 75ºC and then drop to 37ºC. Once the probes were ready 10-12µL of probe was pipetted onto each coverslip and the slide taken from the PBST, dried of excess solution and the fibres placed over the probe. Bubbles were minimised and the coverslip sealed using rubber cement. The sealed slides were then taken to the Top Brite automated FISH and placed on the pre-warmed surfaces. The slides were removed from the machine and placed in a humidified chamber at 37ºC for 24-72h. After this the fibre FISH protocol continues as described in 2.2.15.

2.3 Results

The highly ordered structure of chromatin within the nucleus is plastic, and gene and chromosome relocations can and do occur. In this highly ordered system there would be mechanisms for directing these changes in organisation and alterations in the histone code are potentially involved. Linking alterations to histone code to gene movement requires techniques that can assess both protein and gene sequence positioning within the nucleus. Two techniques used individually to visualise histone modification and gene / chromosome positioning respectively are indirect immunofluorescence (IF) techniques and 2D fluorescence *in situ* hybridisation (2D FISH), however, both require different fixative methods to work. Initial investigation of histone modification pattern distribution within nuclei used a canonical fixation method with 4% PFA and assessment of changes to histone modifications pattern distribution via scoring were performed following conditions known to permit gene relocation. Patterns for several key histone modifications are examined to assess if environmental stress, biological stress or ageing, all factors known to affect gene positioning, effects the global patterning of selected histone modifications within the BB02 strain of *Biomphalaria glabrata*. Further investigations were conducted to see if different approaches could potentially allow 2D FISH fixation using M:AA (3:1) to work more effectively with the

antibodies used for IF, some antibody assays such as αKi67 for visualising proliferating cells work consistently within human cells however, only a few select antibodies or their target epitopes are capable of surviving the harsher fixation method containing acid. Finally, investigations were undertaken to co-localise gene and histone modification markers. To do so development and optimisation of an immuno-fibre-FISH protocol was undertaken. This did allow both gene and histone modification markers to be visualised simultaneously.

2.3.1 Epigenetic alterations induced by environmental stress

Using 4% PFA fixation method for investigating histone modification alterations, experiments began assessing whether heat shock induced stress resulted in quantifiable alterations in histone patterning. Alongside heat shock an assessment was performed to discover whether there were separate responses depending on the form of the environmental stress so snails were also subjected to cold shock to investigate if a difference in histone modification patterns were elicited. $H4K20me^3$, $H3K4me^3$, $H3K27me^3$ and $H3K79me^3$ were all investigated. Two of these modifications, $H4K20me^3$ and $H3K4me^3$ had been previously used for investigation in snail tissue (Odoemelam 2009) whereas the other two modifications, $H3K27me^3$ and $H3K79me^3$, have not previously been used in *B. glabrata*. From these four modifications 5 patterns were discerned overall, although no single modification exhibited any more than 3 patterns in the adult snails.

1. Punctate, patterning exhibiting multiple small foci distributed throughout the nuclei in an even spread.
2. Speckles patterning which showed larger distinctive foci distributed throughout the nuclei that were unevenly distributed.
3. Peripheral, pattern staining that exhibited a continuous stain around the periphery of the nuclei with little to no staining in the centre of the nuclei.
4. Peripheral Foci, pattern staining where distinctive large foci like those in the speckles patterning are located primarily or exclusively at the peripheral of the nuclei.
5. Directional, pattern staining exhibiting heavy staining in one area of the nuclei encompassing part of the peripheral and some of the internal volume,

These are represented in Fig 2.1.

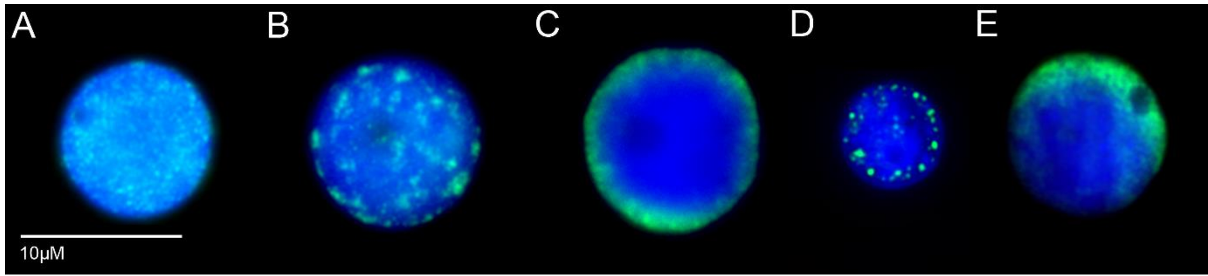


Fig 2.1 Representative images of the 5 patterns discerned from histone modification immunofluorescence techniques on 4% PFA fixed nuclei, images taken from H4K20me^3 (A-C), H3K79me^3 (D) and H3K27me^3 (E). A) Punctate, is made up of multiple small foci spread homogenously throughout the nuclei. B) Speckles, distinct larger foci that are randomly distributed through the nuclei. C) Peripheral, distinct staining around the nuclear periphery. D) Peripheral Foci, distinct larger foci that are predominantly situated near the nuclear periphery. E) Directional, distinct staining on one are of the nuclei encompassing only part of the periphery and interior of the nuclei.

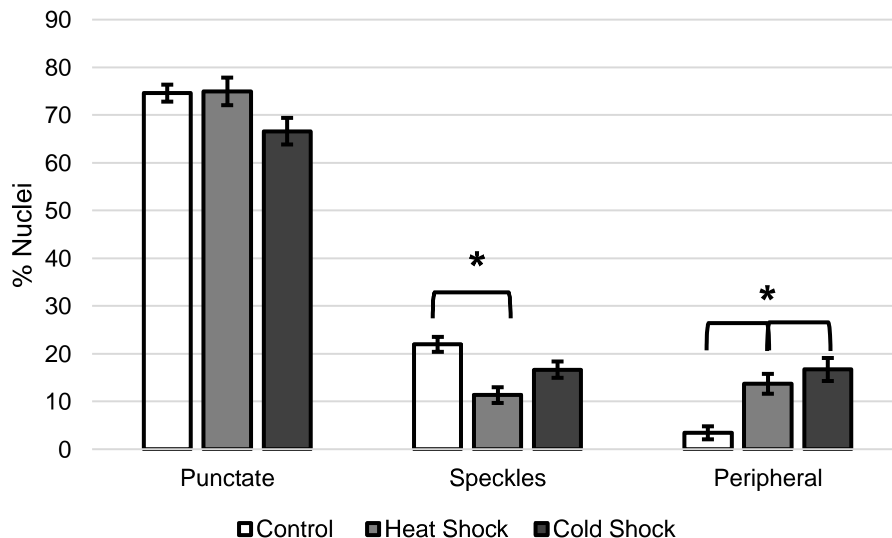


Fig 2.2 Graphical representation of percentage of pattern distribution for H4K20me^3 in 4% PFA fixed nuclei for three states, control, heat shock (32°C) and cold shock (16°C). * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei, in triplicate

H4K20me^3 showed a predominantly punctate pattern of staining in all three conditions however it did show statistically significant changes in the two minor patterns. Speckle staining showed a decrease in both heat and cold shock although it was only significant

during heat shock with $p < 0.01$. This decrease in speckle staining was accompanied by an increase in peripheral staining, which was significant in both heat shock ($p<0.05$) and cold shock ($p<0.01$), although this change in cold shock was also accompanied by a minor drop in punctate as well. Neither of the thermal shocks showed any statistically significant change from one another which may indicate that both are treated the same when the cells try to adapt, these changes are shown in Fig 2.2.

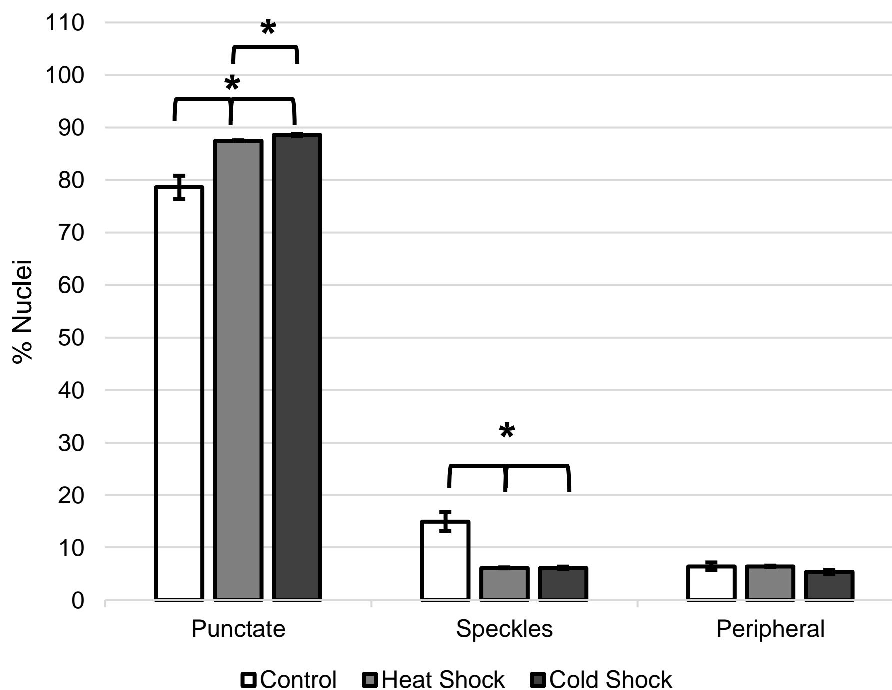


Fig 2.3 Graphical representation of percentage of pattern distribution for H3K4me^3 in 4% PFA fixed nuclei for three states, control, heat shock (32ºC) and cold shock (16ºC). * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei, in triplicate

H3K4me^3 also exhibited a predominantly punctate patterning but showed significant changes in two patterns in response to either thermal shock. In both cases punctate patterning displayed an increase ($p<0.05$) that appeared to be derived from a similar decrease in speckle staining ($p<0.01$). Peripheral staining remained unaffected, however H3K4me^3 is the only modification to exhibit a differential response to cold shock with a very minor increase in punctate ($p<0.01$) in comparison to what was scored in heat shock for, as is demonstrated in Fig 2.3.

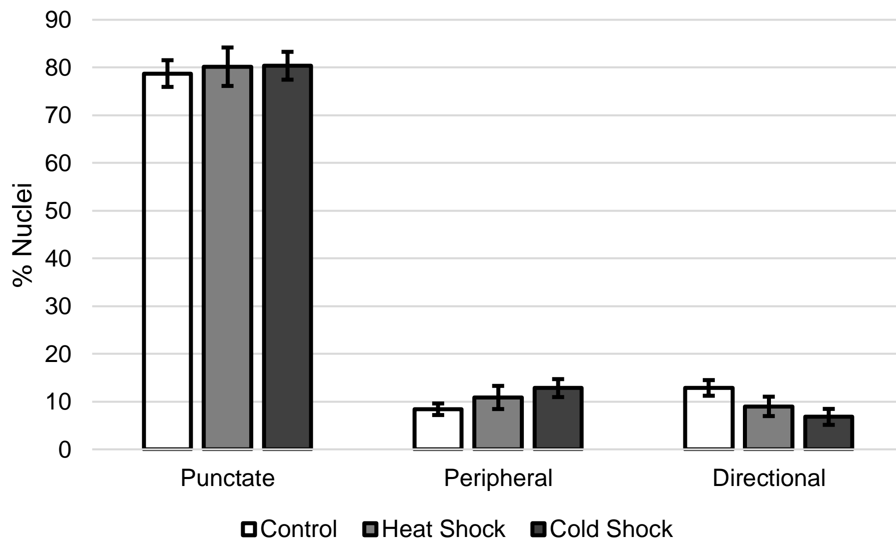


Fig 2.4 Graphical representation of percentage of pattern distribution for $H3K27me^3$ in 4% PFA fixed nuclei for three states, control, heat shock (32ºC) and cold shock (16ºC). * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei, in triplicate

$H3K27me^3$ also showed a predominantly punctate staining pattern with no statistically significant alterations observed as a response to the inducement of thermal shock. At best what can be inferred from the percentages shown is that both heat shock and cold shock may potentially cause a reduction in directional staining in favour for peripheral staining and cold shock may cause a greater reaction, as demonstrated in Fig 2.4.

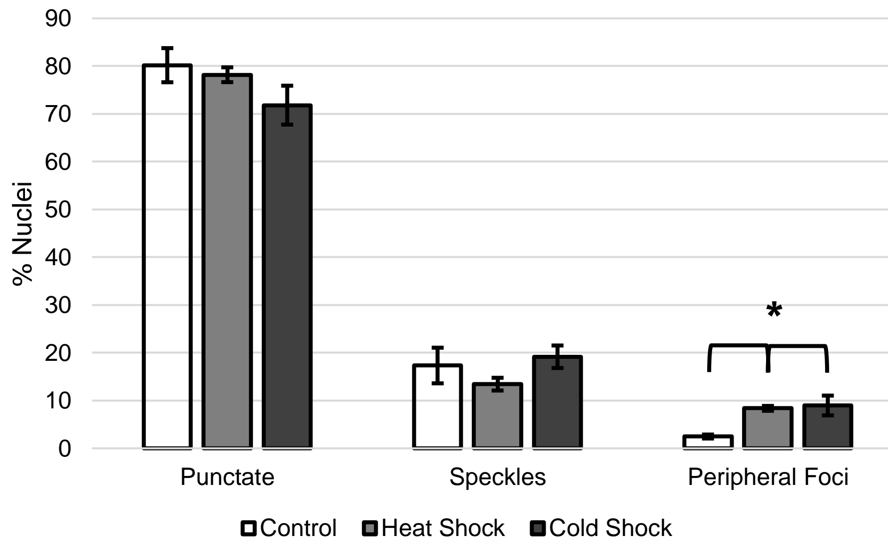


Fig 2.5 Graphical representation of percentage of pattern distribution for H3K79me^3 in 4% PFA fixed nuclei for three states, control, heat shock (32°C) and cold shock (16°C). * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei, in triplicate

H3K79me^3 displays a predominantly punctate staining pattern similar to the previous modifications however, it is the only one to show the peripheral foci pattern and it is this pattern that is most affected by thermal shock. After being subjected to either heat or cold shock the peripheral foci pattern significantly increases over what is found in the control state, p-values = <0.001 and <0.05 respectively. Neither of the other two patterns display any considerable changes to accommodate for this change although one could surmise that in heat shock it may come from a combination of a reduction in punctate and speckles whereas in cold shock it probably comes from a reduction in punctate as demonstrated in Fig 2.5.

2.3.2 Alterations to Histone Modifications as a result of Infection

Schistosoma mansoni can induce multiple gene movement events within *Biomphalaria glabrata* snails upon initial infection (Arican-Goktas *et al*., 2014). *B. glabrata* is most susceptible to infection prior to reaching sexual maturity and as such for these experiments smaller juvenile snails were used and only three antibodies were investigated, H4K20me^3, H3K27me^3 and H3K79me^3. In these experiments to assess if parallels to the heat shock

induced gene movement could be drawn, juvenile snails were either subjected to heat shock at 32ºC for 2h or were 2h post infection with *S. mansoni* miracidia. The $H3K79me^3$ modification was of particular interest during infection as it had been highlighted as a potential target from RNAseq (Knight unpublished) data as being affected post infection. No new patterns were seen in the juveniles in any condition other than those already described in Fig 2.1.

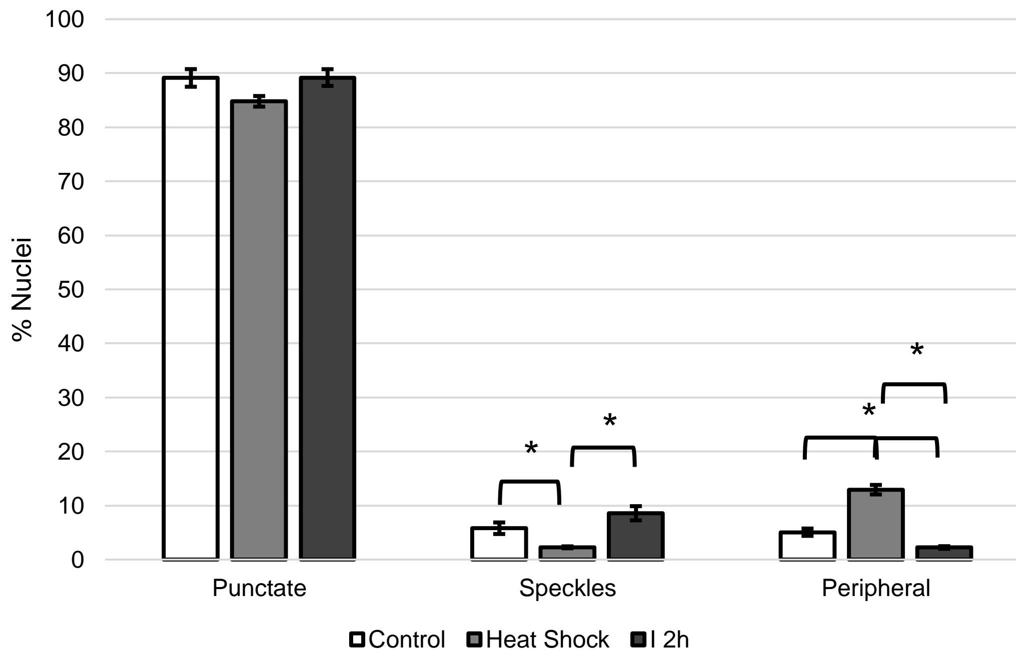


Fig 2.6 Graphical representation of percentage of pattern distribution for $H4K20me^3$ in 4% PFA fixed nuclei for juvenile snails in three states, control, heat shock and 2h post infection. * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei in triplicate.

Fig 2.6 clearly shows a change in histone pattern distribution as a result of environmental and biological stress when compared to the control. Furthermore it also shows that despite both heat shock and *S. mansoni* infection causing alterations in histone patterning the pattern distribution varies significantly between the two stresses with a significant decrease in speckles and concomitant increase in peripheral staining, as seen in the adult snails, as a result of heat shock. Whereas the biological stress of *S. mansoni* infection results in a decrease in peripheral staining and the two stressors significantly differ in both speckle and peripheral patterning.

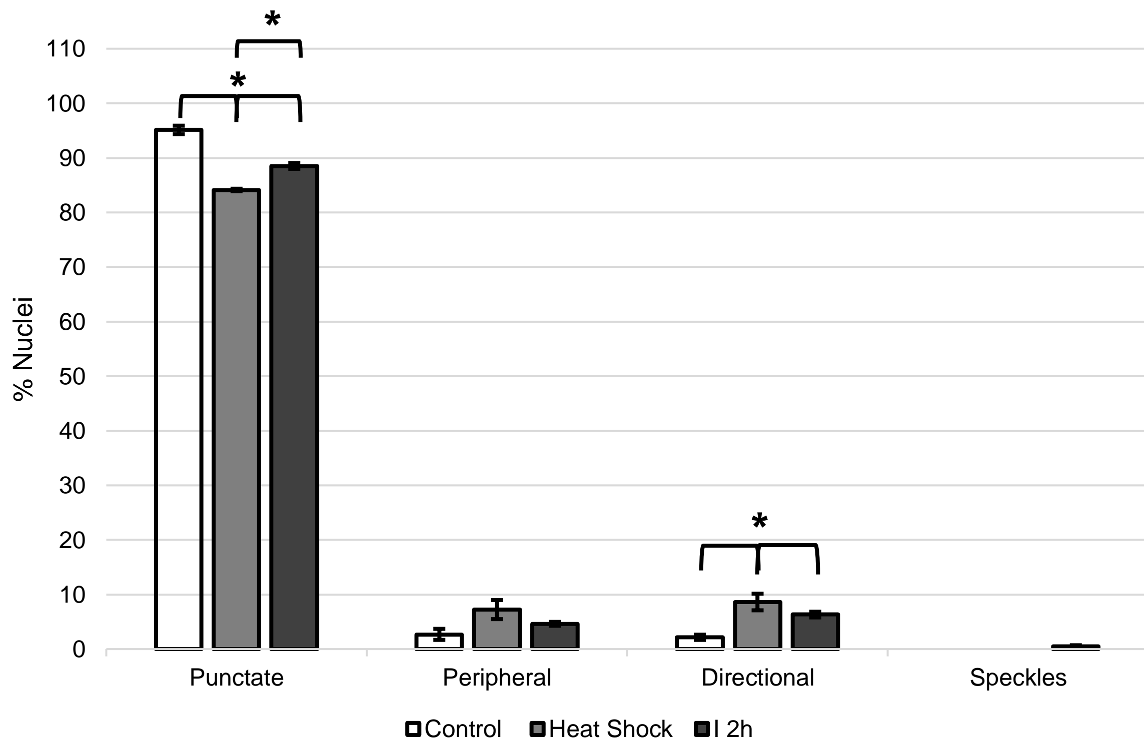


Fig 2.7 Graphical representation of percentage of pattern distribution for H3K27me^3 in 4% PFA fixed nuclei for juvenile snails in three states, control, heat shock and 2h post infection. * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei in triplicate.

As demonstrated in Fig 2.7 H3K27me^3 unlike with the adult snails the juvenile snails exhibit some significant changes after heat shock notably a decrease in punctate staining and increase in directional staining. A similar but less pronounced change is also observable as a result from *S. mansoni* infection. Although there is the same observed change as a result of both environmental and biological stress there is also a significant difference in the number of punctate nuclei when comparing heat shock to 2h post infection, in this case it would appear as if the changes caused by the *S. mansoni* infection are less dramatic in the decrease in punctate staining which is also seen when comparing the peripheral and directional patterns. However, infection also resulted in a new pattern previously not seen using H3K27me^3 antibodies which was speckles, although this was not statistically significant.

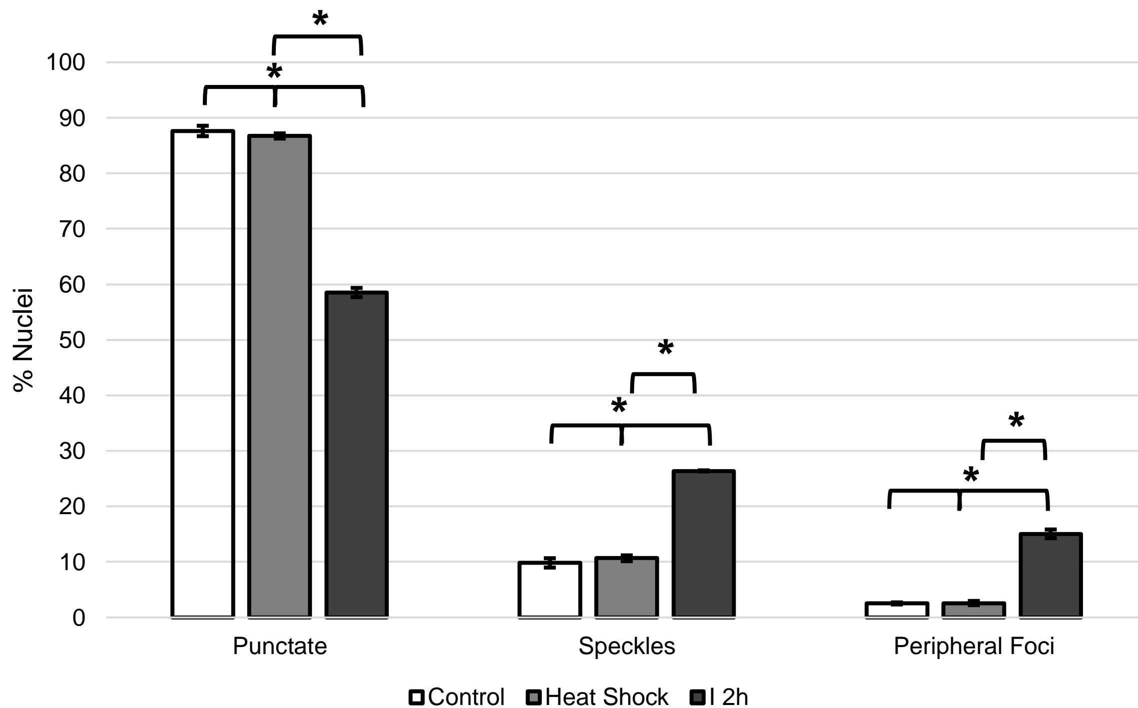


Fig 2.8 Graphical representation of percentage of pattern distribution for H3K79me^3 in 4% PFA fixed nuclei for juvenile snails in three states, control, heat shock and 2h post infection. * denote statistically significant changes in pattern distribution with p-value = <0.05, error bars = ± S.E.M, n = 1000 nuclei in triplicate

Fig 2.8 shows that in juvenile snails, heat shock has no discernible effect on H3K79me^3 pattern distribution. However, the biological stress of *S. mansoni* infection results in significant alterations to H3K79me^3 pattern distribution. There is a dramatic decrease in punctate staining with concomitant increases in both speckles and peripheral foci staining resulting in markedly different pattern distributions from both the controls and heat shock cohorts. This indicates there is something specific to *S. mansoni* infection which either requires alteration to H3K79me^3 pattern distribution or it is potentially something the parasite itself is influencing to happen.

2.3.3 Optimisation of Fixation for IF

Initial optimisation for IF work using M:AA (3:1 v/v), hereafter referred to as the FISH fixative, was to optimise timing for incubations with this fixative for 2D immuno-FISH experiments. An H3K9me^3 primary antibody was employed. Initial experiments assessed the ability of the

antibody to bind the histone methylated group and be visible for three conditions. For primary these were 30min at 37ºC (Fig 2.9A), 1h at RT (Fig 2.9B) and overnight at 4ºC (Fig 2.9C). For the secondary antibody, donkey anti-rabbit (DαR) TRITC, these were 30min at 37ºC (Fig 2.10A), 1h at RT (Fig 2.9) and 4h at 4ºC (Fig 2.10B). The primary antibody incubations were assessed whilst maintaining a secondary incubation at 1h at RT.

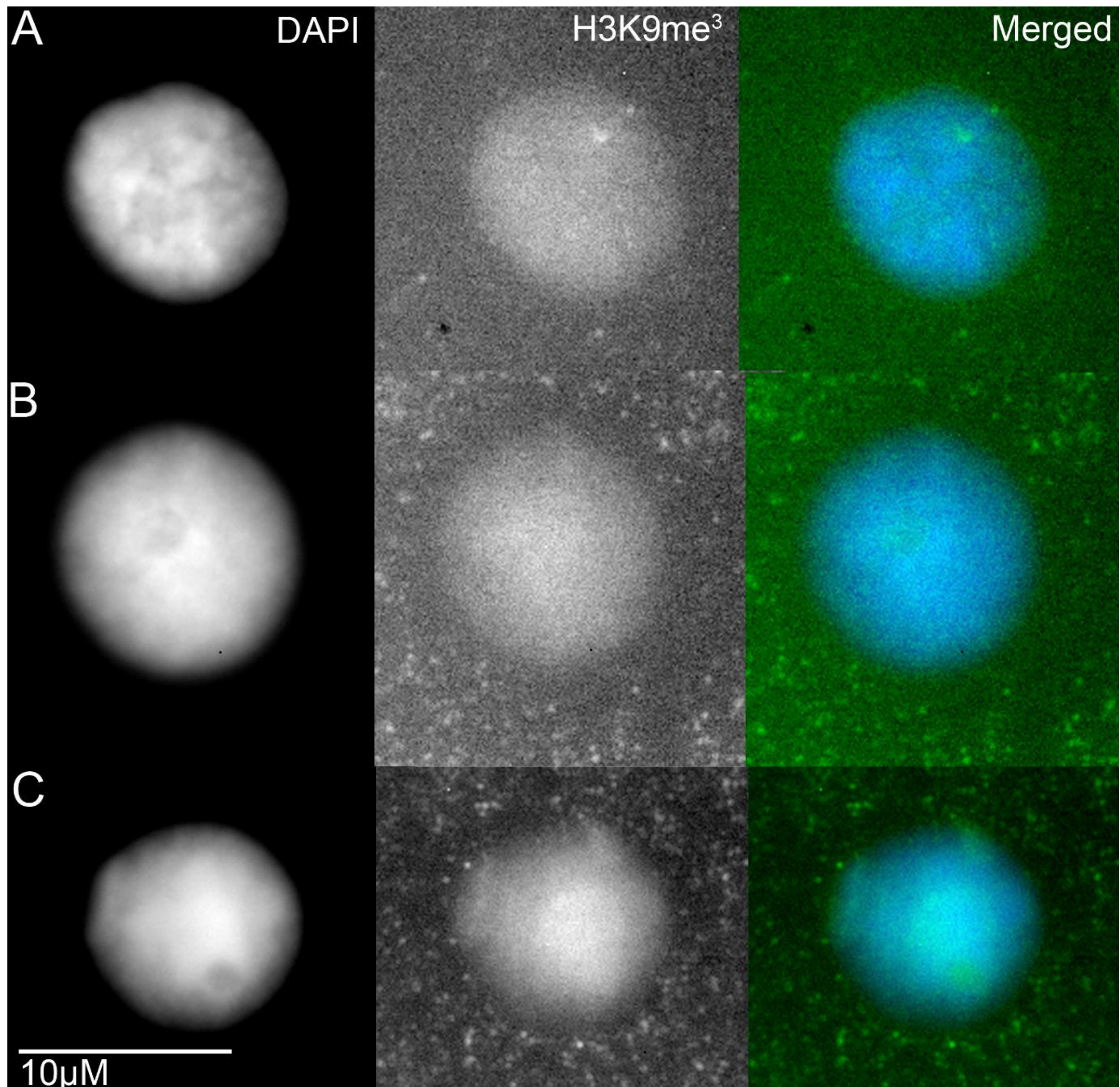


Fig 2.9 Images representative of observations made in M:AA fixed nuclei during the H3K9me^3 antibody optimisation, each image shows a different parameter for the H3K9me^3 incubation while maintaining the DαR TRITC incubation at a standard 1h at RT. H3K9me^3 incubation times varied as follows A) 30min at 37ºC, B) 1h at room temperature, C) overnight at 4ºC. Indicating that an overnight incubation (C) results in the least background.

As is evident in Fig 2.1 the optimal incubation for the H3K9me^3 primary incubation was 4ºC overnight, with an overall reduction in background signal and brighter intra-nuclei staining. Epitopes being revealed overnight has been seen before for nuclear lamins and a specific antibody in human cells (Bridger, J. M. *et al.*, 1993). The next step was to assess whether altering the length of time the DαR TRITC secondary antibody was incubated could further enhance the staining.

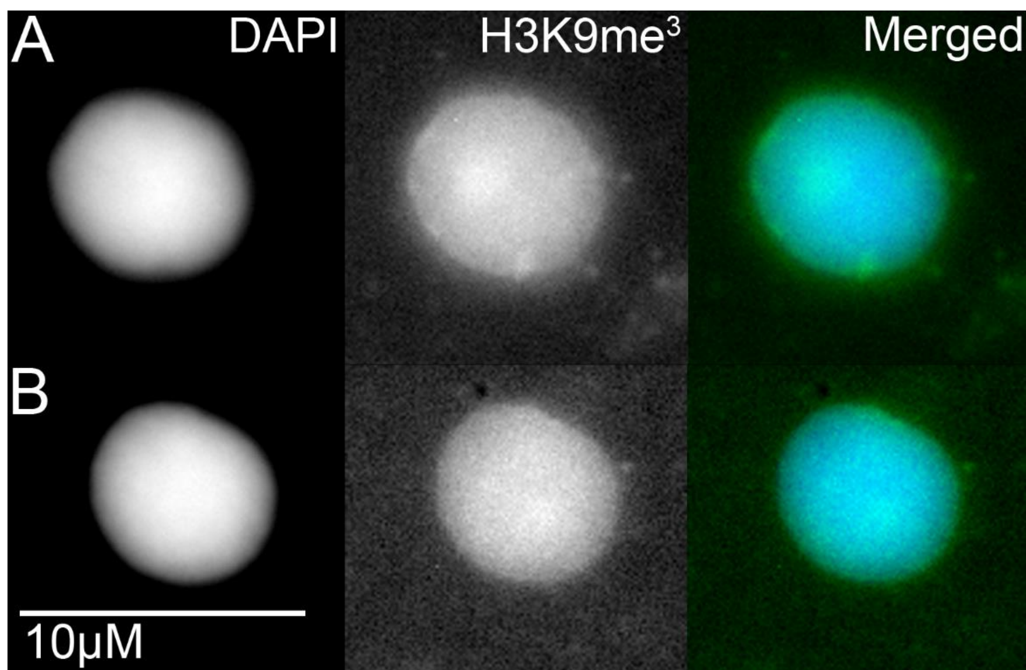


Fig 2.10 The images are representative of what was seen during DαR TRITC secondary antibody optimisation in M:AA fixation while maintaining H3K9me^3 incubation at a consistent 4ºC overnight. A) 30min at 37ºC and B) 4h at 4ºC. These were compared to the 1h RT incubation exhibited in Fig 2.1. No appreciatable change noted in either background or nuclei staining intensity was observed.

Fig 2.10 reveals that there was no discernible improvement between the different incubation conditions for the secondary antibody so utilising a 37ºC for 30min for the secondary antibody as the shortest time was selected. However, even with this optimisation there were significant numbers of nuclei that were negative for any staining pattern over the nascent background which would be unexpected as H3K9me^3 should be a ubiquitous marker in all nuclei (Kim, H. and Kim, 2012). The next step was then to improve the penetration of the antibodies into the snail nuclei. Previous assays using IF and 2D FISH such as the αKi67

staining for proliferating cells are performed after 2D FISH. Therefore a process in the 2D FISH preparation may result in better nuclei staining. The first step after preparing slides for a 2D FISH is the ageing of the slides. This was simple to perform, to assess if allowing them to age naturally at room temperature for either 24h or 48h could improve overall nuclear staining. Following ageing in both time periods there was a reduction in the amount of background fluorescence, although this was most evident in the 48h ageing. The 48h aged slides also showed increased nuclear staining, this is shown in Fig 2.11.

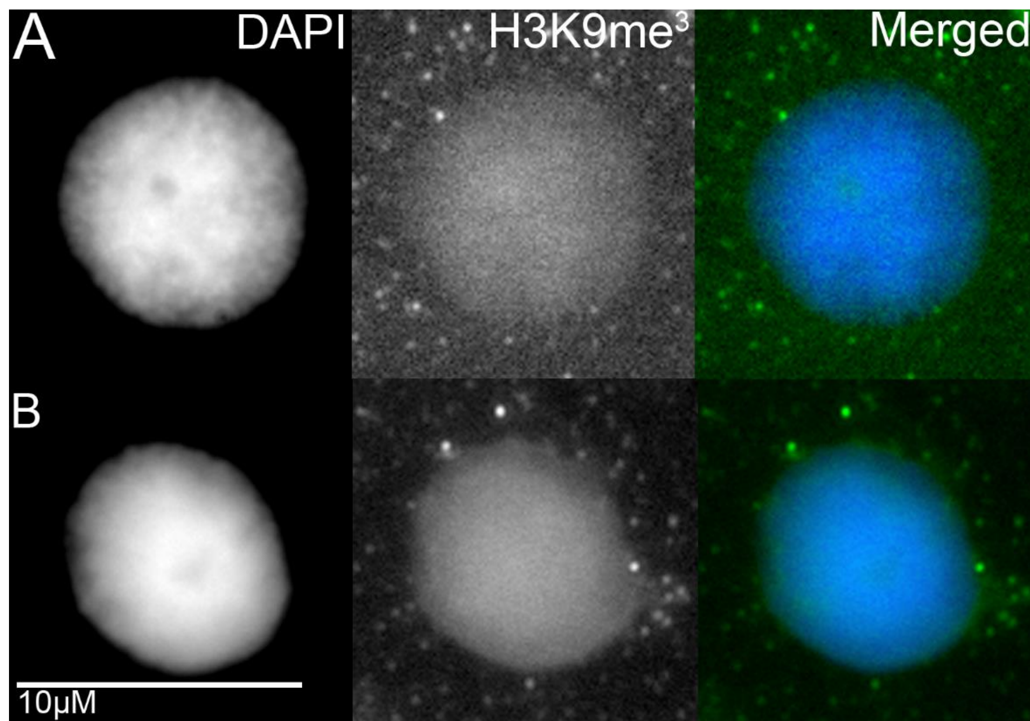


Fig 2.11 The images are representative M:AA fixed nuclei stained using H3K9me^3 primary antibody overnight at 4ºC and DαR TRITC secondary for 30min at 37ºC after the slides had been aged at room temperature for either A) 24h or B) 48h. B, 48h ageing exhibits a reduction in background and increased nuclear staining

Figure 2.11 reveals that there was some reduction in background and more nuclei were positively stained positive for H3K9me^3 with more discernible distributions with both 24 and 48 hr ageing. Ageing in this instance refers to preparing slides allowing them to dry, placing in a container to prevent dust from settling on the slides and leaving them for either 24h or 48h. The ageing of the nuclei had improved overall IF with a reduction in background and increased nuclear staining seen in the 48h aged nuclei.

Another method for improving antibody penetration into the nuclei would be to add a permeablisation step as is required for cross-linking fixatives. Generally, these are not required for alcohol fixations but additional permeablisation may aid antibody entry, it may also assist in revealing epitopes as demonstrated in ChIP on H3K79 methylation states (Steger *et al.*, 2008). The reagents tested were Triton X-100 in a dilution series 0.5-5%, 1% NP-40, 1% Tween 20 and 1% Saponin. Of these only the Triton X-100 revealed any real improvement in nuclear staining and pattern discernibility at a 1% concentration. The final factor that was explored for improving the FISH fixative method for IF was the temperature of the fixative. Typically, fixation is performed cold to slow the reaction of the methanol, which fixes via a combination of coagulation and removal of lipids from the nuclear membrane, resulting in permeablisation. Fixation temperature has also been shown to be important in the optimisation for other imaging techniques (Hobro and Smith, 2017). So an experiment was performed to see if using an ice cold M:AA [3:1] solution would improve antibody staining using the previously established primary and secondary antibody incubation times. This however showed no improvement in antibody staining, instead a reduction of nuclear staining was exhibited with an increase in background staining, and this is demonstrated in Fig 2.12.

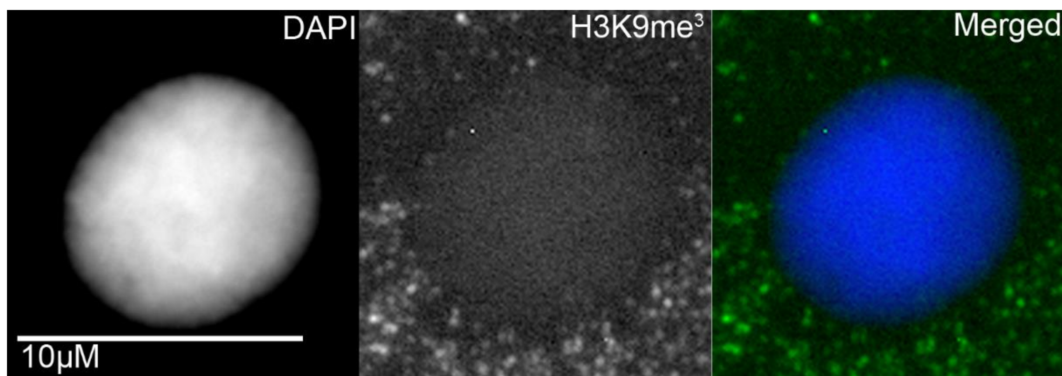


Fig 2.12 Fixation using ice cold M:AA acid (3:1) using H3K9me^3 primary antibody 4°C overnight incubation with DαR TRITC secondary antibody 30min at 37°C incubation demonstrates a reduction in discernible nuclear staining and increased background in comparison to nuclear staining.

Finally, all aspects that improved the IF on the cells in FISH fixative were combined, natural ageing for 48h, 1% Triton X-100 combined with an overnight primary antibody incubation at 4°C and 30min secondary antibody incubation at 37°C. This resulted in an increased number of positively stained nuclei and reduction in background fluorescence.

2.3.4 Pattern discernment and comparison of methanol : acetic acid fixation [3:1] to methanol : acetone [1:1] and 4% PFA fixation methods

Defining and scoring the patterns for comparison to other fixation methods in preparation for assessment of global assessment of changes to histone modifications were performed using a $H4K20me^3$ primary antibody. $H4K20me^3$ is another common marker and the antibody had previously been demonstrated to work in human and *B. glabrata* cells. This antibody was therefore used for the first set of tests to compare differing fixation methods to see how the optimised IF with FISH fixative compared to the previous established fixation methods. Initially, the FISH fixative was used and six variant patterns were identified.

1. Punctate, patterning exhibiting multiple small foci distributed evenly throughout the nuclei.
2. Speckles patterning which showed larger distinctive foci distributed unevenly throughout the nuclei.
3. Peripheral, staining that exhibits a continuous stain around the peripheral of nuclei with little to no staining in the interior of the nuclei.
4. Peripheral Foci, pattern staining where distinctive large foci like those in the speckled patterning are located primarily or exclusively at the peripheral of the nucleus.
5. Asymmetrical, pattern staining where irregular shaped areas of the nucleus exhibit heavy staining while other areas show little to no staining resulting in an asymmetrical appearance.
6. Negative, no staining visible within the nuclei

These patterns are shown in Fig 2.13. An initial count taken for purposes of comparison.

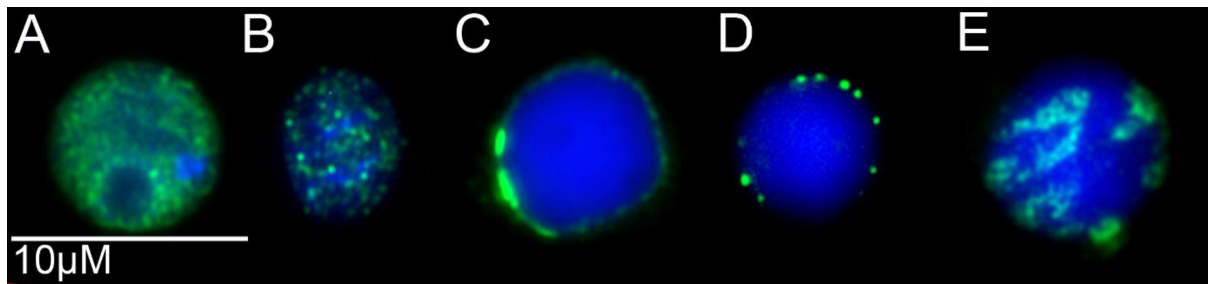


Fig 2.13 Representative images of the discernible patterns identified using H4K20me^3 primary antibody and DαR TRITC secondary antibody staining using M:AA (3:1 v/v) fixation. A) Punctate, multiple small foci distributed throughout the nucleus in a homogenous spread B) Speckles, larger distinctive foci distributed throughout the nucleus that were not as homogenously distributed C) Peripheral, staining that exhibited a continuous stain around the peripheral of the nucleus D) Peripheral Foci, distinctive large foci like those in the speckled patterning are located primarily or exclusively at the peripheral and E) Asymmetrical irregular shaped areas of the nucleus exhibit heavy staining while other areas show little to no staining.

The objective of optimising the FISH fixative for IF is to allow immuno-FISH be conducted on the samples. This would allow histone modification distribution and gene positioning to assessed in tandem, as it is likely that the changes to histone modifications is an initial step to chromatin reorganisation and occurs prior to the gene movement to either guide or initiate the changes required. As such two slides were assessed, one from control snail and the other derived from a heat shocked snail to investigate if changes to pattern distribution could be discerned. The results of the initial test are shown in Fig 2.14.

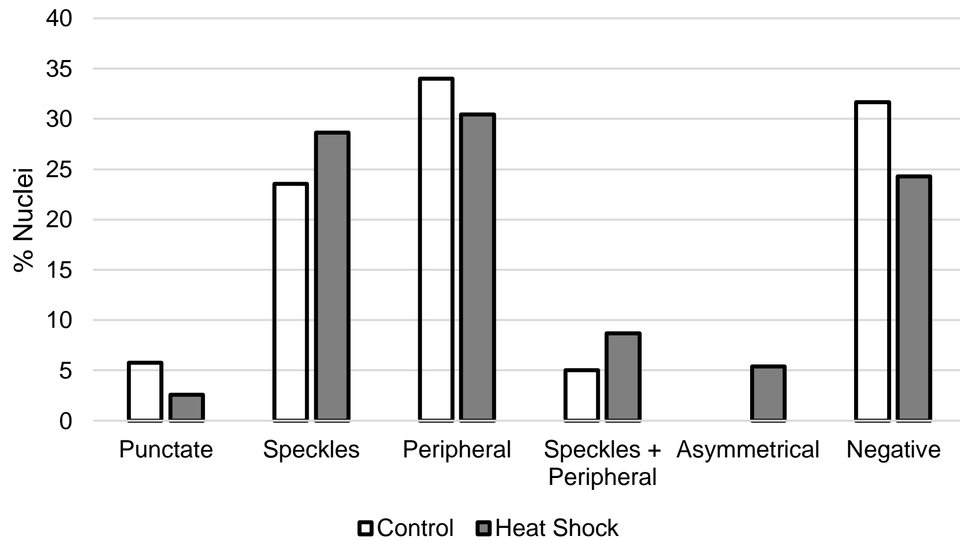


Fig 2.14 Graphical representation of the percentage of each pattern discerned during initial count using $H4K20me^3$ staining using M:AA (3:1) fixation with and without heat shock to assess if global pattern changes could be discerned using this method. Control (n= 259 nuclei) and heat shock (n= 391 nuclei), no repetition was done for initial assessment of method viability.

As can be seen from Fig 2.14 there were still a significant number of negative staining nuclei even after optimisation of FISH fixative for IF, for histone modifications it seemed that the FISH fixation was less than ideal but not unusable. The next step was to test the more common fixation methods, methanol:acetone (M:A) (1:1 v/v) and 4% formaldehyde solution made up from paraformaldehyde powder to avoid the methanol used in concentrated formaldehyde solutions to minimise polymerisation of the formaldehyde in to paraformaldehyde. As with the FISH fixations were performed at room temperature. With both the canonical fixation methods negative staining diminished significantly to <2% of nuclei and this remained consistent in later experiments for multiple antibodies. Due to this consistent low level of negative staining it could be attributed to either of the two antibodies not penetrating the nuclei, the nuclei on the edge of the slide not being covered by the parafilm or similar other issues that could result in incomplete stain of all nuclei. When combined with the ubiquitous nature of modifications such as $H4K20me^3$ and $H3K27me^3$ it is more likely that there was incomplete coverage than negative staining. The initial experiment with M:A fixation only displayed four distinct patterns of distribution, punctate, speckles, peripheral and asymmetrical.

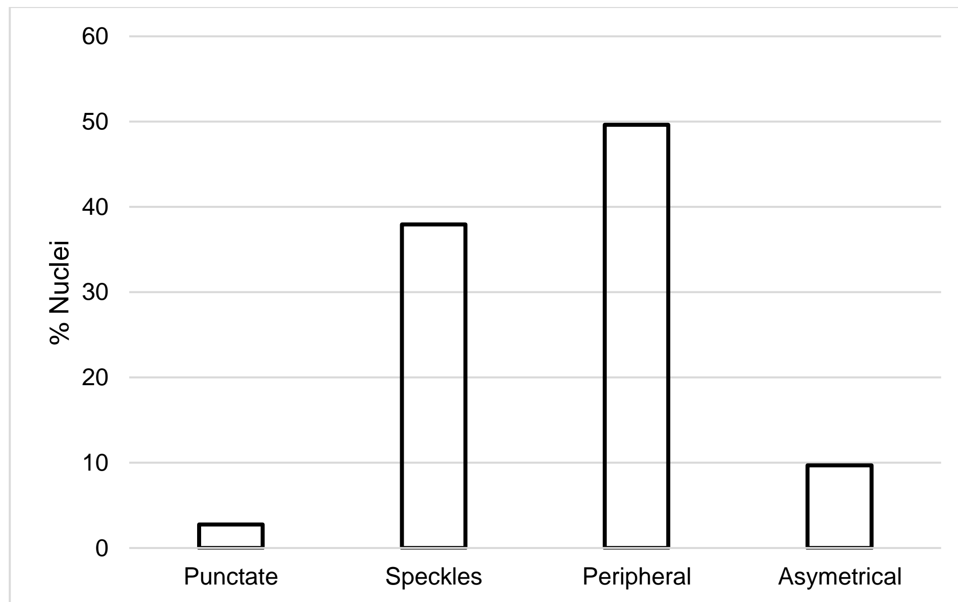


Fig 2.15 Graphical representation of the percentage of each pattern discerned during initial count using H4K20me^3 staining on control snails using M:A fixation (n= 403 nuclei).

Fixation using M:A fixation more commonly used for IF resulted in clearer staining with four clearly defined patterns. The 4% PFA fixation resulted in three easily identifiable patterns, punctate, speckles and peripheral as well as showing significantly different pattern numbers which were clearer than in either the M:A or the FISH fixative. As such although M:AA fixation has been used successfully in the past for certain IF antibodies and it does work to an extent with histone modifications it is best to utilise the cross-linking fixation methods. This does mean that 2D FISH and histone modification IF could potentially be done together but validation of specific antibodies would be required to denote that the acid fix had not affected the functionality of the antibody.

2.3.5 Fibre FISH Lysis Buffer Optimisation

Being able to perform both FISH and IF on cells together would allow for better visualisation of the interactions between histone proteins and genes however, due to the abundance of histones, if we take 916 Mb (Adema *et al.*, 2017; Gregory, 2003) as our estimate, there could be up to 124,000 of each histone within each nucleus. Therefore 3D FISH would likely be extremely difficult to analyse as well as requiring more specialised equipment than normal IF. Hence the objective of this experiment was to develop a protocol for performing Fibre-

FISH on *B. glabrata* cells which would allow for the visualisation of gene and histones allowing for co-localisation of specific histone modifications using 2D methods. This would also serve as an intermediate step for the development of chromatin immunoprecipitation (ChIP) qPCR experiments allowing for confirmation of association of gene and histone protein prior to full scale experimentation.

Biomphalaria glabrata cells and nuclei are notoriously hard to lyse requiring extended times in hypotonic solution over human cells but, are also particularly fragile under certain conditions which means making adjustments is extremely difficult to gauge. Since initial cell lysis had already been optimised for 2D FISH isolating nuclei was not an issue but multiple methods for nuclear lysis had to be explored. The protocol that was initially followed was one written by Prof Beth Sullivan (Sullivan, 2010). The initial step was to optimise the time required in the lysis buffer. Due to *B. glabrata* nuclei being comparatively small the initial density used was based on the protocols optimised for *Drosophila melanogaster* which utilised 2.2×10^5 cell/mL (Sullivan, 2010). The basic buffer using the lowest concentration of urea (0.2M) was used and a time series of 12 - 16 minutes was executed. Using this alone resulted in minimal lysis during later time stages of 15 and 16 minutes. Similar methods for lysis were considered. Another method for generating fibres was to use a 0.1-0.4M NaOH with 30% methanol solution to soften the nuclei and help fix the fibres to the slide using manual combing as described in (de Barros *et al.*, 2011), this method resulted in the loss of all nuclei on the slide so was discounted.

Further optimisation of Sullivan lab lysis buffer was performed using the same cell density and two time points, 12min and 16min, with 0.3M and 0.4M urea versions of the buffer respectively. The 16min time point at 0.4M urea showed the greatest lysis but this was still only approximately 5% of the nuclei. Alterations were made to the cell density trying a range of cell densities from 2.2×10^5 down to 1.0×10^4 using the 0.4M lysis buffer for 16min the lower density range showed the best lysis but the 1.0×10^4 had too few nuclei present on the slide to work with and 5.0×10^4 although showing improvement in nuclear lysis, this lysis was minimal only creating bundled chromatin next to almost complete nuclei while other nuclei had swollen but not lysed as demonstrated in Fig 2.16.

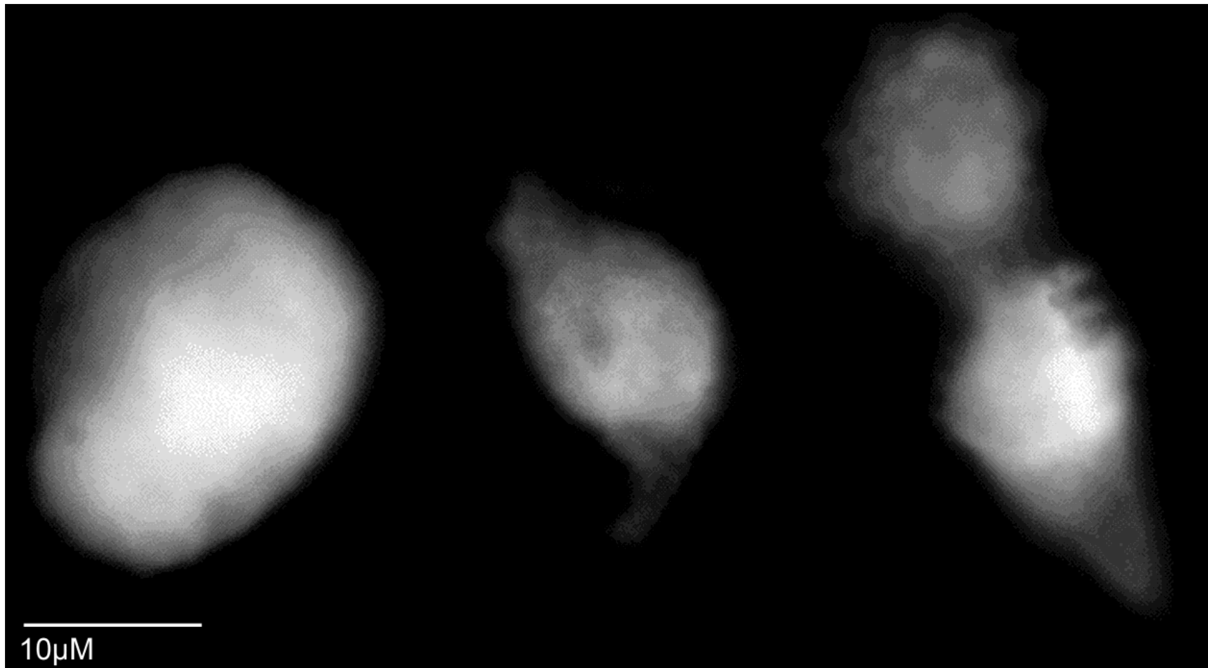


Fig 2.16 Representative image of the 16min time point using the unaltered nuclear lysis buffer from Prof Beth Sullivan's lab. This demonstrates that no complete lysis was achieved, only either swelling of nuclei but with no lysis or minor lysis where minimal chromatin is extracted as bundles just outside the nuclei without forming any distinctive fibres.

The next step was to use $5x10^4$ cell density and decrease the salt concentration to make it even harsher in an attempt to cause more lysis. The NaCl concentration was dropped from 0.5M to 0.2M and lysis was performed for 16 and 20min, while the unmodified lysis buffer was also run for 20 and 25min respectively to see if increasing lysis duration worked. By altering the salt concentration better lysis was achieved although the chromatin was still found in bundles rather than clearly defined single chromatin strands as is exhibited in Fig 2.17.

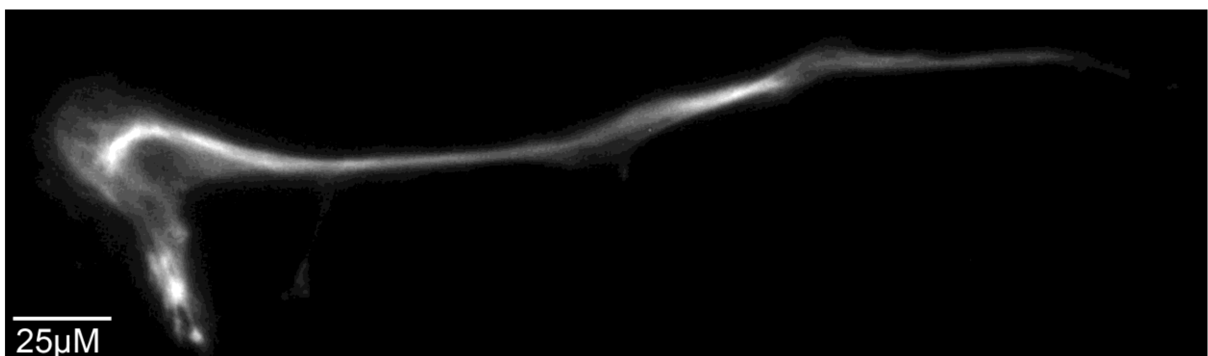


Fig 2.17 Representative image of nuclear lysis after using a 0.2M NaCl, 0.5M urea modified lysis buffer for 20min. Although lysis has occurred the nuclei is still showing mainly chromatin fibre bundles rather than single chromatin fibres

Another nuclear specific lysis buffer that is normally employed for ChIP was developed by the Gozani lab (50mM Tris pH8.0, 10mM EDTA pH8.0 and 1% SDS (w/v)). This had previously shown some promise in cross-linked ChIP optimisation for *B. glabrata* nuclei and was a more stringent lysis buffer than that suggested by the Sullivan lab. RIPA buffer (150mM NaCl, 5mM EDTA, 50mM Tris, 1% NP-40 (v/v), 0.5% sodium deoxycholate (w/v) and 0.1% SDS (w/v)) was also tested alongside the Gozani nuclear lysis buffer each at two time points 10 and 15mins. Both initially seemed to show improved lysis but, with the Gozani nuclei lysis buffer showing the greatest improvement as represented in Fig 2.18, ultimately this was not repeatable and the best consistent results had still come from the Sullivan lab buffer.

Fig 2.18 Representative image taken from initial Gozani nucleus lysis buffer at 15min of lysis, here more completely lysis of the nuclei was achieved and single chromatin fibres were identifiable, this however was not repeated in later experiments using this lysis buffer.

Returning to the Sullivan lab buffer further alterations were made this time to the urea concentration again as the increase to the suggested maximum of 0.5M had had the best effects on lysis so far as well as experiment with surfactant changes. 2M urea, 5% Triton X-100 (v/v) (Sigma Aldrich, UK), 0.5% SDS (w/v) (Sigma Aldrich, UK), 0.5% sodium deoxycholate (w/v) (Sigma Aldrich, UK) were all tried for 20mins of lysis with and without manual combing to see the effects additional force had on the nuclei and production of chromatin fibres. This revealed that 2M urea and the addition of manual combing both had positive effects on lysis, although the greatest improvement was from the addition of manual

combing. The substitution of Triton X-100 as the surfactant to SDS or sodium deoxycholate also seemed to improve lysis but, DAPI staining following either of these treatments had reduced intensity in comparison to previous fibre staining so were ruled out. To assess if any further improvement could be made a 7M urea solution was used as well but, no further enhancement in lysis was observed. So a final test was run using the modified lysis buffer, as outlined in 2.2.12, using 20, 25 and 30min lysis in conjunction with manual combing, shown in Fig 2.19. 15min incubation exhibited increased overall lysis of nuclei but fibres were still mainly contained within bundles not yielding the single chromatin fibres that would be needed. 20min incubation in the lysis buffer resulted in a greater number of single chromatin fibres but there were still several bundles. 30min lysis resulted in the greatest number of lysed nuclei and more consistent generation of single chromatin fibres.

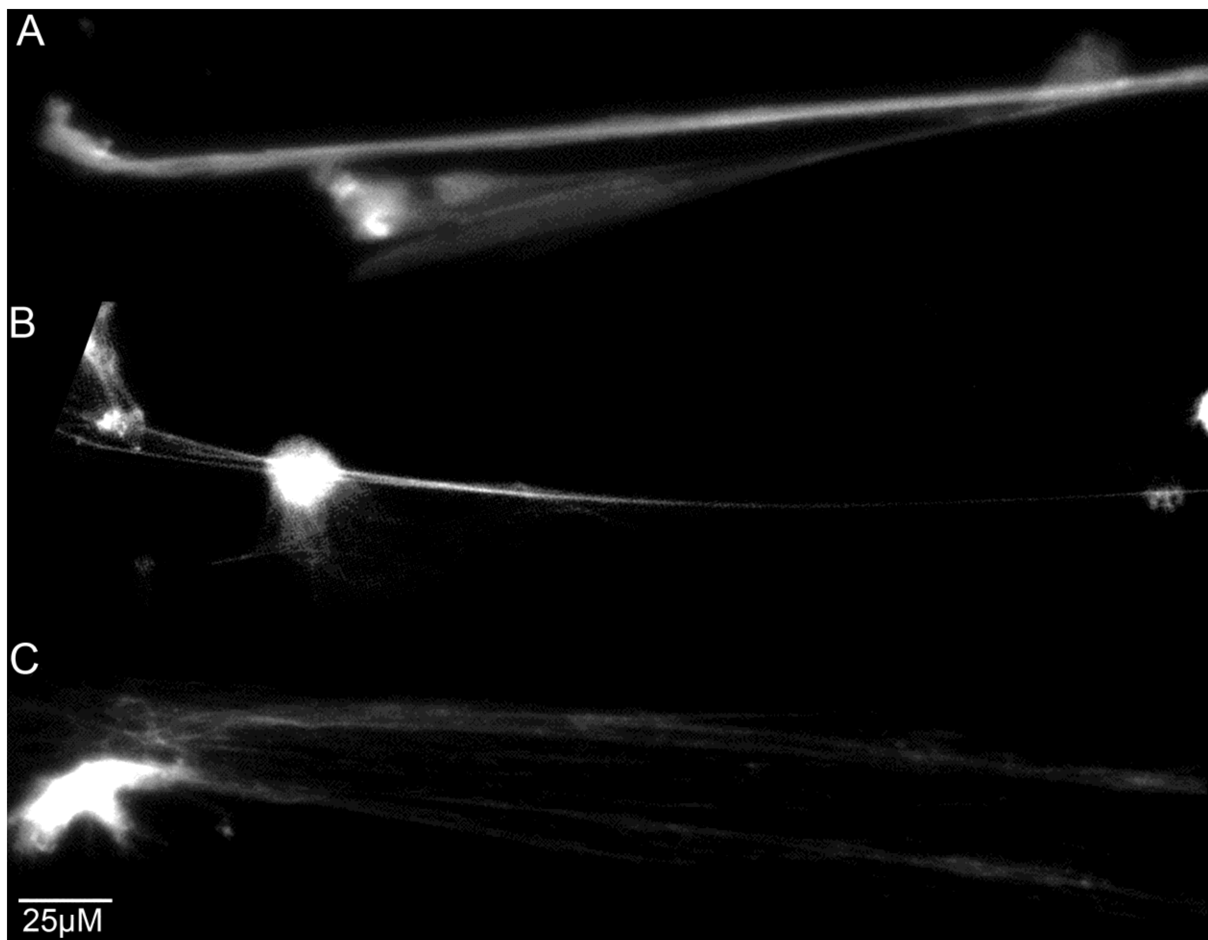


Fig 2.19 Representative images of 2M urea adjusted Sullivan Lab lysis buffer at incubated for A) 15min, B) 20min and C) 30min followed by manual combing. A) Shows improved lysis but, still mainly bundled fibres, B) shows improved lysis and more single chromatin fibre formation, C) shows more single chromatin fibres and a better spread of fibres and overall on the slide more nuclei showed consistent lysis.

2.3.6 IF on Chromatin Fibres

Following optimisation of the lysis buffer solution indirect immunofluorescence staining using the H3K79me^3 primary antibody and DαR TRITC secondary antibody was performed on the fibres. The H3K79me^3 which had previously been shown to work in IF on *B. glabrata* and remains relatively consistent in staining was used at a 1:400 dilution and DαR-TRITC secondary was used at a 1:200 dilution. This proved successful at a 4x10^4 cell/mL density. The results of which can be seen in Fig 2.20.

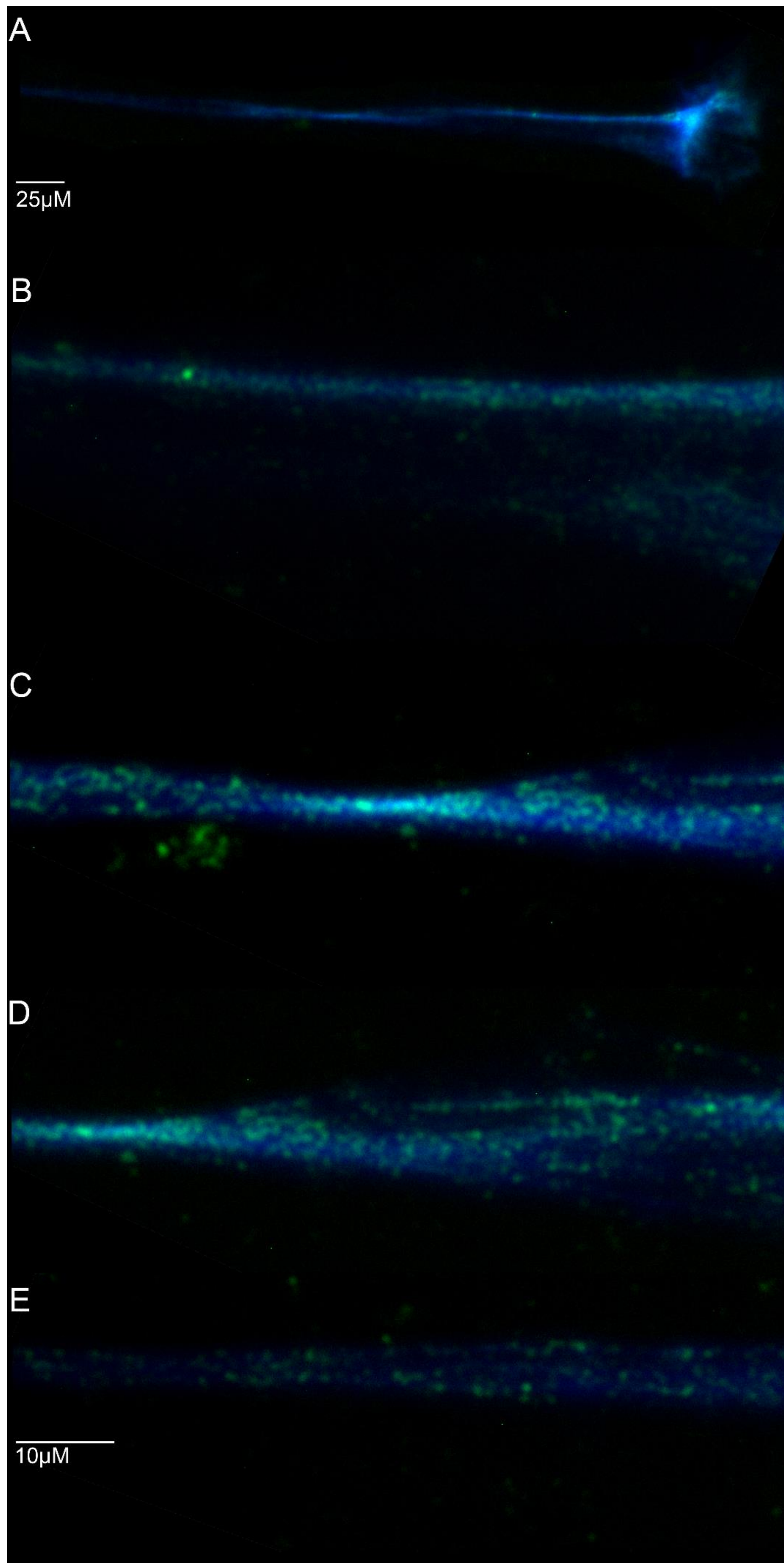

A
25μM
B
C
D
E
10μM

Fig 2.20 Representative images of IF for histone modification H3K79me^3 on chromatin fibres extracted from *Biomphalaria glabrata* cells derived from the ovotestis. A) The full length of the lysed nucleus, B-E) 1000x maginification of the chromatin fibre bundle demonstrating the H3K79me^3 staining. Although the lysis this time resulted in mainly chromatin bundles some single fibres can be distinguished.

Although the fibre extraction is incomplete and most remain in bundles which is not ideal it does show a proof of concept that with further refinement this could be used to associate gene and histone modifications visually prior to moving into targeted ChIP-qPCR work. This is the first time IF has been performed on chromatin fibres derived from *B. glabrata* and shows that the lysis process used is not causing damage to the histone protein visualised. After this the fibres were once again fixed using 4% FA to fix the IF signal in place and FISH was attempted on the fibres as per 2.2.14. This did result in dulling of the IF signal as well as loss of fibres and unfortunately no FISH signal was found using a Strepavidin-Cy5 conjugate 1:200 dilution (Jackson Labs).

2.3.7 FISH on Fibres

Having managed to successfully generate fibres and stain them for IF signal, the lack of FISH signal was unfortunate but, due to IF signal already requiring the Cy3 filter an unfamiliar streptavidin-Cy5 conjugate had been used and one that was not fully optimised for FISH on *B. glabrata* nuclei. As a result to see whether fibres generated could be targeted by FISH it was performed alone in the absence of IF as a proof of concept. This highlighted several issues with the FISH process, fibres are very fragile and easily lost due to mechanical force so washes have to be done static and simple movements could potentially cause loss or breakage of fibres. The FISH process requires several changes of solution normally and removal of either coverslips or parafilm which sometimes necessitate manual removal. With this it was evident that even without going through IF protocol first several fibres were damaged or lost. Another problem was the mountant normally used did not hard set so even after the final coverslip was in place some movement could occur which potentially damages the fibres. Although through this a few images were captured that potentially showed gene signal on the fibre, Fig 2.21, it would require further optimisation to be able to run through the whole process.

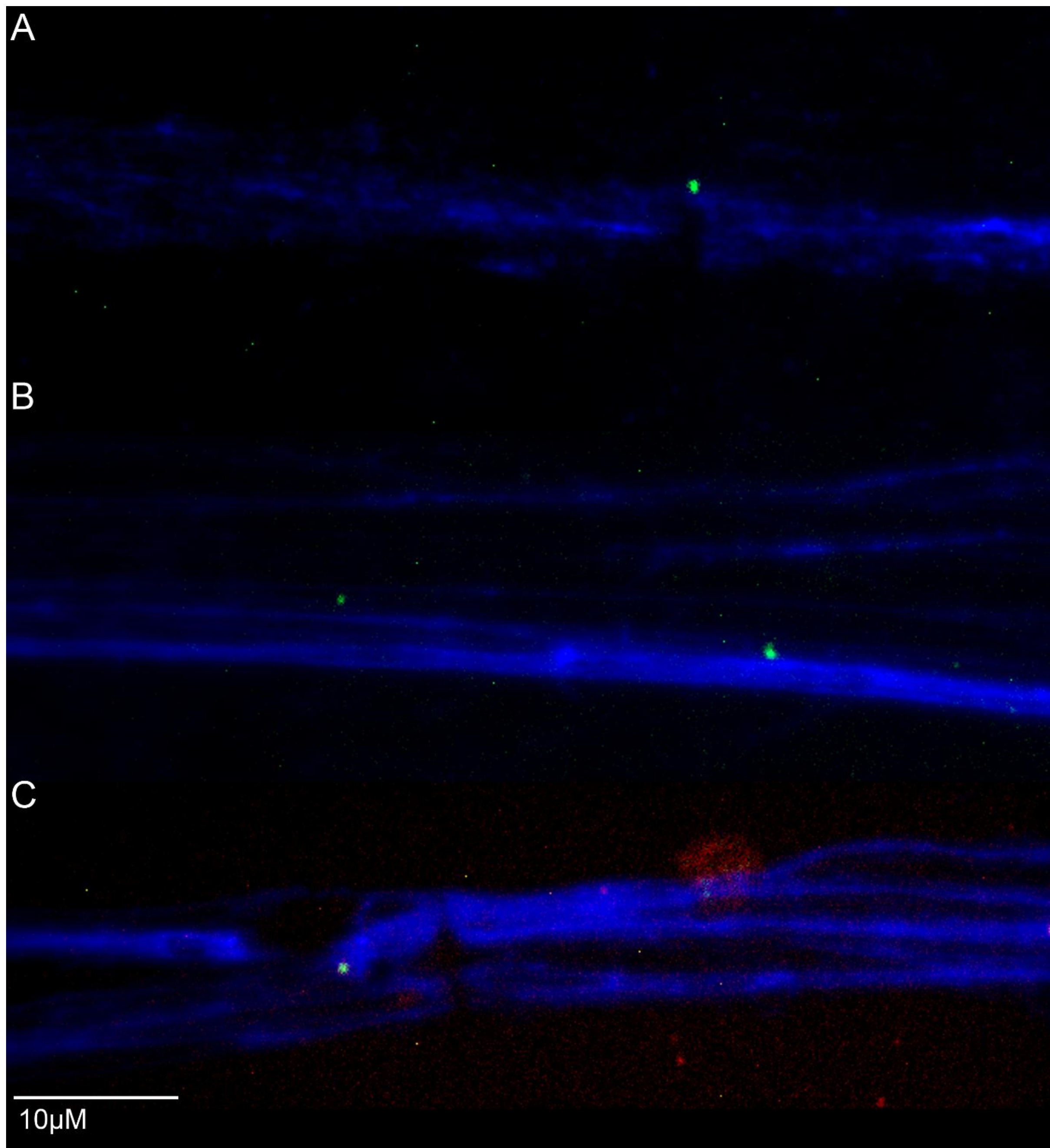


Fig 2.21 Images that show *hsp70* loci signal in fibre bundles following Fibre FISH. Green in these images is the *hsp70* loci, blue are the chromatin fibre bundles. A) Displays some breakage and disruption to the fibre. B) Representative of unbroken chromatin fibres although the signal is contained within a bundle. C) Chromatin bundles that have been slightly damaged but still shows *hsp70* loci close to the start of separating into single fibres.

2.3.8 Fibre FISH

Optimising the protocol required altering the cell density once more as it had become clear that several fibres would be lost throughout the process due to washes, movement and solution changes. This also needed to be readdressed as the initial optimisation was utilising buffer alone to lyse the nuclei resulting in a $1.0x10^4$ - $4.0x10^4$ cell density being used, generally around the $4.0x10^4$ mark, with the addition of manual combing, increased lysis time and optimised buffer it was likely a greater density could be used without issue. Further experiments found that a $1.0x10^5$ to $4.0x10^5$ cell density provided ample lysed cells with minimal to no overlap.

Optimisation of the protocol was required to limit the amount of potential damage the slides were exposed to during the process. The simplest way to achieve this was to combine two steps, the denaturation of the chromatin and the application of the coverslip. Using the Top Brite automatic FISH system (Resnova, Italy) no formamide denaturation solution is required as it uses the formamide in the hybridisation mix the probe in dissolved in to denature the chromatin as it is warmed up. This also eliminates one step whereby the solution or movement could damage the fibres.

The final issue was the mountant used for the fibres, in this instance a hard set formulation was found that cured at room temperature for 15min which eliminated the potential for post mounting damage to occur. As such the whole protocol was undertaken with the greater cell density, H3K79me^3 primary antibody was used and a Goat αRabbit Alexa Fluor 488 (ThermoFisher, UK) secondary was used to allow the FISH to be done using an optimised Strepavidin-Cy3 conjugate (Jackson Labs, UK) for visualisation of the gene signal.

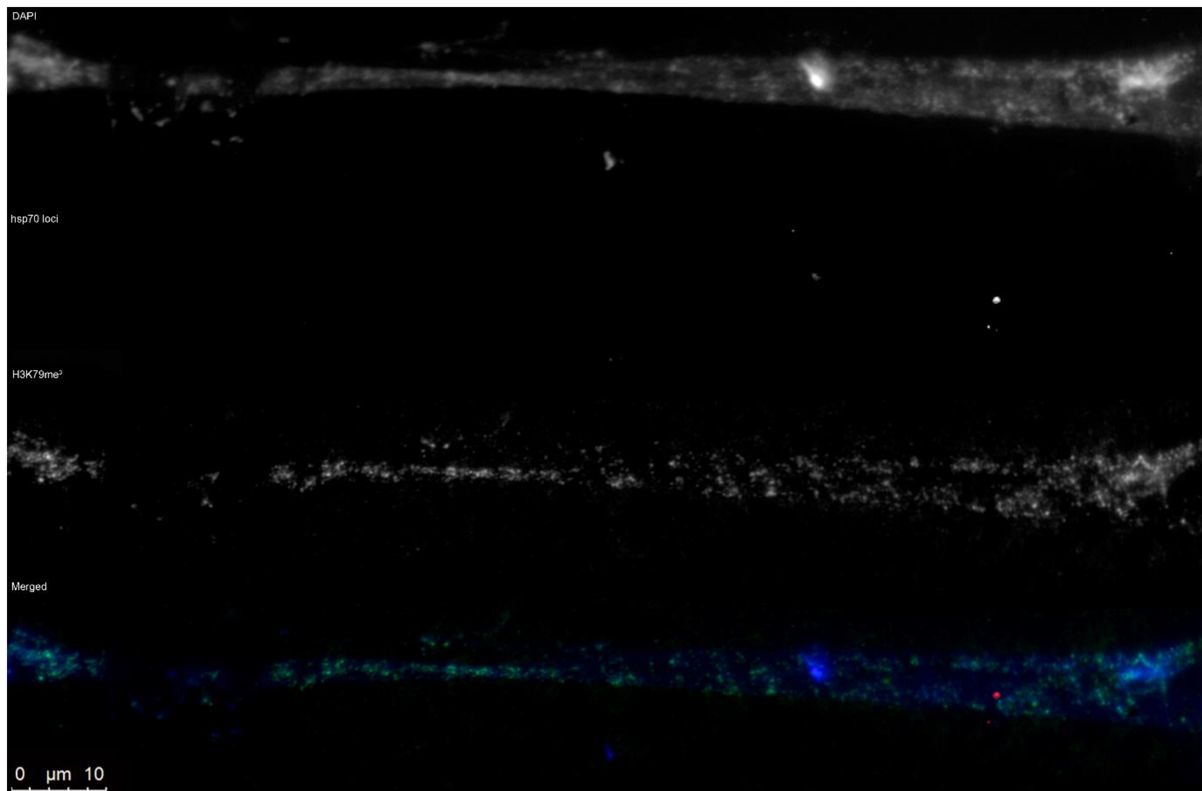


Fig 2.22 An image showing both IF of H3K79me^3 in green and FISH of *hsp70* loci in red on a chromatin fibre bundle in the merged image and with the channels separated for ease of distinguishing the the IF and FISH signals.

Unfortunately single clearly defined fibres were not found with both the IF and FISH signal however, it does show a chromatin fibre bundle with both gene and histone modification signals. With some further refining this technique can certainly be used to visually assess whether there is a correlation between a gene and a histone modification and using image analysis software potentially even quantify that relationship to an extent. It would certainly however act as a confirmatory step prior to ChIP-qPCR to assess whether there is a correlation between the two that should be picked up by ChIP provided appropriate primers and antibodies are available.

2.4 Discussion

Indirect immunofluorescence is one of the basic techniques used for investigating structures and proteins within nuclei but trying to combine this with other techniques to get a greater overview of protein chromatin interactions as with histone modifications and gene loci is

difficult due to the comparative difficulty of optimising existing techniques for new organisms and need for specialised equipment. Typically, such interactions would require the use of 3D FISH and confocal microscopy to visualise. 2D FISH in comparison requires no more specialised equipment than IF does and is quicker to carry out and easier to analyse than 3D FISH but using a different fixation method from IF. Taking this into consideration optimisation of IF to work with the M:AA fixation used in 2D FISH was a logical step forward. Certain antibodies, such as the αKi67 used to test for proliferating cells had been successfully used after FISH previously showing that it was at least possible to perform IF on M:AA fixed cells.

After much experimentation and comparison with the more typical fixation methods used for IF it became apparent that although certainly possible to do IF for histone modifications on M:AA fixed cells it was not ideal. It resulted in a large proportion of nuclei with negative staining, which later experiments using 4% PFA showed just was not the case, and pattern discernment was made harder due to increased background, reduced intensity of staining and quite possibly alteration due to the harsh acidic fixation that was potentially affecting the epitopes the antibodies were targeted against. This meant that although possible it would likely not provide any definitive results and any result of IF for these histone modifications done on M:AA fixed cells would be suspect and need to be verified by other methods. This is especially true when comparing the original data collected from the $H4K20me^3$ which showed minimal to no punctate patterning, but in PFA fixed cells punctate makes up the majority of patterning. This could indicate that such small but numerous foci are the most susceptible to damage via the acidic fix hence why the large number of negative stained nuclei and apparent increase in patterns exhibiting larger foci or the asymmetrical staining pattern which could have been a result of damage to punctate patterning.

Not shown here is IF for a non-histone protein that was investigated for, HP1α, this is a histone associated protein involved in many processes. This protein was indicated from work related to *S. mansoni* infection, as playing a role in a stress response. This protein was clearly present in large foci as was expected in nuclei that had been fixed using the M:AA fixation method. However, later attempts at identifying it by using either the M:A or 4% PFA fixations failed with four separate antibodies. This raises an interesting question as to whether the M:AA fixation caused a false positive reaction or whether the acidic fix that destroyed / effected the epitopes for histone modifications worked in the favour of the HP1α epitopes by revealing them for antibody to attach to, similar situations have been found with

ChIP and H3K79me^3 (Steger *et al.*, 2008). Considering the staining seen in the few cells with a positive response look very similar to that seen in human cells in the literature it may be the case that the acidic methanol:acetic acid fix could have some advantages in specific cases. To explore this however would require greater investigation outside the scope of the current investigation that was specifically looking for a way to visualise both a gene loci and histone modification together using a combined 2D FISH - IF approach.

The next stage was to assess whether stress induced changes to chromatin organisation would be detectable with a global approach using IF alone. To do this it was determined that a global stressor was necessary in this case heat shock was already known to affect chromatin organisation to a degree as it can induce the movement of the *hsp70* loci and can cause previously resistant snails to become susceptible to *Schistosoma mansoni* infection (Ittiprasert, Wannaporn and Knight, 2012). It had also been shown to affect histone in *Drosophila melanogaster* either by increase phosphorylation (Dyson, Thomson and Mahadevan, 2005) or by triggering removal of nucleosomes form active sites (Zhao, Herrera-Diaz, and Gross, 2005). This indicated that heat shock likely had quite a significant effect on the snail and one that could hopefully be seen in changes to histone patterning as the *hsp70* gene loci was moved. Four histone modifications were chosen, H3K27me^3 and H4K20me^3 are both well-known and documented induces of gene inactivation, likely one of the response to sudden stress would be the down regulation of genes, especially in the case of heat shock those that coded for proteins that would be particularly susceptible to the increased temperature. H3K4me^3 is a known marker for gene activation, such as is known to occur with *hsp70* during heat shock and finally H3K79me^3 another marker for gene activation but, like HP1α previously mentioned also highlighted to become upregulated during *S. mansoni* infection.

H4K20me^3 showed no real change in the majority of the nuclei but we did see a change shifting from a speckle pattern to one more concentrated at the periphery, this could indicate a greater shift towards activation of the internal genes while ensuring the more peripheral genes were properly locked down maybe because they are the ones most susceptible to heat shock or are genes that have been moved to the periphery due to being more susceptible and while under stress the rearrangement ensures there is a lower risk of proteins produced by those genes being transcribed and damaged. Conversely, the gene activation marker H3K4me^3 also exhibits a decrease in speckle staining but increases the number of punctate nuclei showing a more global internal distribution of gene activation

markers rather than being concentrated in distinct foci. In all states however there is minimal peripheral staining which is more common in the gene inactivation markers of H4K20me^3 and H3K27me^3 which supports the notion that a switch to peripheral staining for H4K20me^3 may be to further consolidate gene inactivation of those peripherally located gene loci. H3K4me^3 is the only marker that showed any statistically significant change between heat shock and cold shock but, this was a very minor increase in the number of punctate nuclei, this could indicate that more gene activity is required to combat cold shock over heat shock but, since it is the only one to show any significant change further investigation would be required to assess if there is indeed a significant difference in epigenetic response to differing temperature extremes.

H3K27me^3 showed no significant changes in response to thermal stress, this may be because although it is a gene inactivation marker part of that association is accumulation of the H3K27me^3 marker because the gene has become inactive so it is also a consequence of inactivation not just a cause for inactivation. Such a marker therefore may not be rapidly changed in response to stress as it has other functions in relation to genome organisation. It would be interesting however, to assess how this marker may be changed as a result of a longer term stress, such as living in a hotter environment since this has been shown to cause the F1 generation of resistance snails to become susceptible even after being return to their preferred temperature (Ittiprasert, Wannaporn and Knight, 2012). Finally, the H3K79me^3 showed only minor variation with an increase peripheral foci staining, which could be indicative of more active genes moving towards the periphery as H3K79me^3. This may be counter intuitive since the periphery is generally associated with gene inactivation but, H3K79 methylation in yeast also denotes a change from active euchromatin with H3K79 methylation staining and SIR protein silenced telomeric regions which would likely be moved to the periphery (Ng *et al.*, 2003; Ng *et al.*, 2002). With H4K20me^3 staining more prevalent at the periphery in heat shock and linked with its role with increased chromatin compaction (Evertts *et al.*, 2013) it is not strange that a marker known to border silenced regions like H3K79me^3 may also be drawn to a more peripheral position.

Following the creation of baseline in the adults during environmental shock three of the antibodies were tested in the juvenile snails. H3K79me^3 was selected due new data showing that it was upregulated in infection, it was therefore a significant marker to proceed with to assess how closely heat shock induced gene movement mimicked the *S. mansoni* infection induced gene movement. H3K27me^3 was retained as a standard marker for gene

inactivation although it had not shown any significant change in the adults it is also a marker indicated to change due to age so may show a different response in the juveniles. H4K20me^3 had already exhibited change in the adult snails so could be used as a benchmark for changes in a gene inactivation marker if H3K27me^3 proved to show no significant difference as with the adult snail.

H4K20me^3 did show that there was indeed a change from the adult baseline in comparison to juvenile controls. However, the response to heat shock observed was exactly the same as that in the adult snails, the decrease in speckles for a concomitant increase in peripheral staining. The infection response was exactly the opposite with a decrease in peripheral staining with an increase in speckles, this could indicate a global decrease in gene suppression and chromatin compaction which would likely facilitate increases in expression of target genes such as *hsp70*, *hsp83* and *actin* (Arican-Goktas *et al.*, 2014; Ittiprasert, Wannaporn and Knight, 2012). It would also indicate that although both *S. mansoni* infection and heat shock induce the movement of the *hsp70* loci that the response elicited within the nuclei for both movement differs. This was particularly true when you examined the H3K79me^3 markers which showed absolutely no change between the control and heat shock juvenile snails but, dramatically different changes between the control and infected juvenile snails. The reduction in punctate foci with an increase in peripheral foci and speckles would seem to indicate a switch from a more diffuse spread of gene activation to more targeted hubs where genes have either been marked to become more active or reorganisation of the chromatin due to gene movement has resulted in more concentrated pockets of H3K79me^3 staining in the nucleus. This would seem to support the fact that *S. mansoni* is using some epigenetic signalling mechanism to manipulate the host cells to make it more hospitable.

H3K27me^3 also showed some changes in both heat shock and infection within juvenile snail with decreases in the number of punctate stained nuclei and increases in directional staining. This is in contrast to there being no statistically significant change in the adult snails following heat shock. However, unlike with H3K79me^3 and H4K20me^3 the alterations noted in H3K27me^3 pattern staining were the same in both heat shock and infection, although the response in infection could be described as being somewhat diminished in comparison with that seen in heat shock. This would seem to indicate that alterations in H3K27me^3 may be less important in infection than it is in heat shock or that fewer genes are targeted for silencing during infection than in heat shock.

These results show that it is possible to see alterations in epigenetic markers that may be related to gene movement. They also show that although two stressors, heat shock and infection, may elicit similar responses such as *hsp70* gene loci movement the effect they have on the epigenome will not necessarily be the same. The next steps were to develop techniques for combining FISH and IF to investigate the interplay of histone modification and gene movement at a level where both gene and histone modification can be linked. For this a Fibre-FISH protocol was optimised for work with *Biomphalaria glabrata*.

Working with *B. glabrata* tissue and cells posed many issues when developing protocols optimised to them. While the cells are covered in polysaccharides which can make lysis and DNA extractions difficult they also prove to be extremely hardy under the correct conditions. During optimisation of the Fibre-FISH protocol it became evident that in developing protocols using *B. glabrata* nuclei there is a fine balance between stringency and efficacy that has to be walked to ensure that the nuclei extracted are fit for purpose. A typical issue faced is the choice of surfactant used. Non-ionic surfactants such as Triton X-100 and NP40 are not always stringent enough without prolonged incubation times to cause the desired lysis. Conversely anionic surfactants such as SDS even at low concentrations such as 0.5% were too stringent destroying the fibres and structures that the protocol required to remain, although this may prove more useful in extractions where maintaining structures are less important such as in cases where cross-linking fixation is used, such as that used in ChIP which maintains the protein bindings not just the natural bounds.

Ultimately, the protocol required the combined approaches of two separate protocols, a chemical lysis to soften the nuclei membranes and mechanical combing, to result in the final lysis and spooling of the chromatin fibres. This unfortunately, although it does result in greater lysis also seems to result in less consistent lysis with formation of chromatin bundles being achieved rather easily but, causing separation out down to the single chromatin fibre to occur less readily. It is likely a skill to develop the necessary technique with the combing to increase the occurrence of single chromatin fibres for assessment. It should also be noted that fixation of the antibodies prior to proceeding to FISH did result in some reduction in the brightness of the antibody. Whether this was due to incomplete fixation allowing for some of the antibody to be removed in later washes, damage to fluorophores due to denaturation in FISH or a combination of the two it does mean that using this method may not be applicable for weaker signals or it may require a sandwhich approach to the IF to

improve signal or maybe even do so after the FISH. There are several ways that could improve the signal from the antibody staining if need be.

The development of this technique does however, solve one of the biggest issues faced when running a ChIP-qPCR experiment. That is the uncertainty of whether a histone modification is associated with the gene of interest or not. With the combination of both FISH and IF available within the Fibre-FISH technique one can visualise the approximate relationship of gene signal and histone modification. With many ChIP ready antibodies also applicable for use in IF you can check to see if the antibody works and if it is near or within the gene of interest, this removes the uncertainty factor of gene-protein association when optimising the ChIP experiment. It can also allow for relative measurements to take place of where in the gene signal the histone modifications are. This along with the knowledge of the sequence used for in the creation of the gene probe one can estimate where the modifications may be within the gene allowing for more targeted development of primers for ChIP-qPCR. Finally, using consistent exposure times when taking images, software could be used or developed to measure relative intensity or number of signals contained within the gene signal. This would allow for an estimate of changes in histone modification presence as a confirmatory test to add credence to ChIP-qPCR data or as a method of quantification in of itself once validated.

In summation it is clearly demonstrated that these antibodies, though not designed to work in the snail, due to the highly conserved nature of histone modification across species do work in the new model. There is also clearly exhibited differences in histone modification pattern distribution following a stressor such as heat shock which is a known to potentially induce gene relocation. However, though similar responses are caused by infection in the juvenile there are clear differences in nuclear responses most obvious of which is demonstrated in $H3K79me^3$ pattern distribution which is relatively unchanged after heat shock but significantly different following *S. mansoni* infection. Although ultimately immuno-FISH was not possible a new protocol was developed for the first time in the snail for immuno-Fibre-FISH to allow for future assessment of the co-localisation of gene signal and histone protein modification markers.

Chapter 3: Investigating the effects of epigenetic and genome reorganisation inhibitory drugs on *Biomphalaria glabrata* resistance to *Schistosoma mansoni* infection

3.1 <u>Introduction</u>

It is well established that DNA is not randomly distributed within interphase nuclei (Cremer, T. *et al.*, 1982) and that it in fact forms into distinct chromosomal territories to which they can be consistently and reliably mapped (Croft *et al.*, 1999). The same has been shown with genes being consistently mapped to the same region within nuclei (Arican-Goktas *et al.*, 2014; Szczerbal, Foster and Bridger, 2009) and when induced activation occurs the genes are moved to another non-random nuclear location (Arican-Goktas *et al.*, 2014; Szczerbal and Bridger, 2010). This movement is even associated with upregulation of the moved genes (Arican-Goktas *et al.*, 2014; Szczerbal, Foster and Bridger, 2009). In particular within the freshwater snail *Biomphalaria glabrata* it has been shown for the first time that gene movement precedes upregulation of gene expression (Arican-Goktas *et al.*, 2014). One method by which this directed gene movement is hypothesised to occur is via an actin-myosin motor (Mehta *et al.*, 2010; Dundr *et al.*, 2007; Chuang *et al.*, 2006) similar to that used in cytoplasmic movement. Although the role of actin and myosin as a nuclear motor may be debated (Bridger 2011), it is evident that both protiens do have roles within nuclei which can impact on gene activation (Sokolova *et al.*, 2018; Mehta *et al.*, 2010).

2,3-Butanedione monoxime (BDM) is a known inhibitor of myosins (Ostap, 2002; Soeno, Shimada and Obinata, 1999) and its use interrupts actin-myosin interactions even within nuclei (Mehta *et al.*, 2010). It has also been previously shown to prevent gene movement from occurring in a heat shock model of *Biomphalaria glabrata* (Arican-Goktas, 2013). By inhibiting the nuclear myosins investigations can be undertaken to elucidate the role gene movement has in the upregulation of target genes like *hsp70*. This would potentially inhibit the non-random nuclear relocation of the gene while permitting any changes to epigenome which would signal for that relocation to occur. Allowing for the isolated effect of gene relocation to be explored and indicate if halting gene movement in the host *B. glabrata* alone is enough to increase resistance to infection by the parasite *Schistosoma mansoni* as well as any changes to gene expression that may result from the prevention of gene movement.

It is also well established that epigenetic changes, such as that to the 'Histone Code' (Kühn and Hofmeyr, 2014; Jenuwein and Allis, 2001), can affect gene transcription rates. The two most common forms of modification made to histones are methylation and acetylation. In particular the addition of an acetylation mark is indicative of gene activation (Eitoku *et al.*,

2008; Agalioti, Chen and Thanos, 2002) and in particular the H4K16ac is linked to transcriptional activation (Zippo *et al.*, 2009).

Since histone acetylation is easily reversible and have key roles in gene regulation they would be perfect targets for an invading organism to exploit and due to their role in cancer there have been several drugs developed to target either acetylation, histone acetyltransferase inhibitors (HATi), or deacetylation, such as histone deacetylase inhibitors (HDACi). Three drugs were selected to interfere with changes in acetylation, remodelin a known NAT10 inhibitor (Wu *et al.*, 2018), anacardic acid which inhibits Gcn5 and p300 HAT family members (Ghazifard *et al.*, 2019; Eliseeva *et al.*, 2007) and sodium butyrate which is a HDACi (Monneret, 2005; Davie, 2003). With these drugs it was investigated whether inhibiting either the histone acetylation or histone deacetylation would affect the overall resistance of *B. glabrata* to *S. mansoni* infection. This could potentially narrow down potential targets within the excretory secretory products (ESP) from the parasite that could be used for treatment especially if one or more was found to have functions that affected the acetylome of the host.

The likely mechanisms of action during infection is that upon infection the parasite induces changes in the host cells via ESPs. These cause alterations to the epigenome which is the trigger for alterations to chromatin organisation which nuclear motors facilitate by non-randomly reorganising the chromatin as dictated by the modification to the epigenome. This change in chromatin organisation results in alterations to gene expression with upregulation of target genes such as *hsp70* which the parasite requires to improve survivability in the host and continue its lifecycle. With this approach two points during initial infection by the parasite are being targeted. The motors themselves using BDM, preventing relocation of chromatin from occurring and potentially preventing upregulation of a target gene, *hsp70*, and preventing alterations to the epigenome by inhibiting changes to the histone acetylome.

3.1.1 Aims

- Verify *S. mansoni* induced gene movement is not strain specific
- Investigate the effects of BDM treatment of *B. glarata* on susceptibility to infection and gene expression
- Assess the effects of inhibiting changes to the acetylome on *B. glabrata* susceptibility to infection

3.2 Methods

3.2.1 Cell Suspensions from *Biomphalaria glabrata* tissue for FISH

Cell suspensions were made from a single ovotestis of a *B. glabrata* juvenile snail (≤0.5cm in diameter). The shells were crushed and the ovotestis excised using needle nose tweezers and singly placed in a 0.5mL of a 0.05M potassium chloride (KCl) solution inside a sterilised 1.5mL microcentrifuge tube. The tissue was macerated until no large tissue clumps remained using a tissue grinder (Axygen), another 0.5mL of 0.05M KCl solution was added and then incubated for 30min at RT. The suspension was spun at 200g for 5mins and the supernatant discarded. The cells were then resuspended in the fixative, which consisted of methanol:acetic acid in a 3:1 ratio, which was added dropwise with constant agitation until 0.5mL of fix was added and with a further minimum incubation of 10min at RT. Samples then were spun at 200g, the supernatants discarded and fixative added dropwise once again with constant agitation and incubated for 10min at RT, this was repeated at least once more. The final addition of the fixative was used until the solution looked a watery white colour, generally around 100µL. Once cells were fixed in suspension and stored at -20ºC. After prolonged storage however, samples were spun and fixative replaced.

3.2.2 2D Fluorescence *in situ* Hybridisation

Slides were prepared by dropping 20µL of M:AA fixed cell suspension onto a wet slide from height and the excess liquid drained before drying on a slide dryer set to 37ºC. The slide was then aged for 48h at room temperature or artificially aged by placing at 70ºC for 1h. The slides were taken through a dehydration series of 70%, 90% and 100% ethanol spending 5mins in each before being dried on the slide dryer. Probes were derived from the BB02 and BS90 bacterial artificial chromosome (BAC) libraries encoding for the *actin*, *ferritin*, *hsp70* and *myoglobin* genes.

Once the FISH probe was denatured, see section 2.2.10, the slide was denatured by placing them in a 70% formamide in 2X SSC pre-warmed to 72ºC and incubating them at temperature for 90sec. Immediately after incubation the slides were immersed in ice-cold

70% ethanol for 5min after which they were transferred to 90% and 100% ethanol for 5min each and the slides dried. As the slides were drying 22mm x 22mm coverslips were placed on the dryer as well, one for each slide to pre-warm them for probe addition. Once slides were dry and coverslips warmed 10-12µL of the denatured probe was pipetted onto the coverslip and the slide placed over the cover slip and pressed down. Air bubbles were removed using guided pressure and the coverslip sealed to the slide using rubber solution (Weldtite) and placed in a humidified chamber at 37ºC. Time was critical as the probe cannot be permitted to excessively cool, as such the slides were prepared one at time and the probe was not added to the next slide until the previously one was sealed and in the humidified chamber. Once the probe was added the slides were incubated for 12-72h.

After incubation the rubber seal and coverslips were removed and the slides washed thrice in 2X SSC at 42ºC for 5min each. The excess liquid was drained off and 100µL of 4% bovine serum albumin (BSA) (Sigma Aldrich, UK) in 2X SSC was added to the slides and covered with parafilm and incubated at 37ºC in a humidified chamber for 20mins. The 4% BSA was removed before the addition of 150µL of streptavidin conjugated to Cy3 (Jackson ImmunoResearch) in 1% BSA in 2X SSC (1:200 Dilution), covering with parafilm and incubating at 37ºC for 30mins. The slides are washed in 2X SSC for 5min, followed by 1X PBS with 0.1% tween 20 (Sigma Aldrich, UK) for 1 min and finally rinsed for 1min in 1X PBS. Finally the slides were dipped in sterile water, excess liquid drained off onto tissue paper and then counterstained with DAPI [1.5µg/mL] (Vectashield anti-fade mountant, Vector Laboratories) and a 32x22 glass coverslip placed to cover the nuclei.

3.2.3 Gene Positioning Analysis

Images were taken using an Olympus BX41 fluorescence microscope and processed for image analysis using adobe photoshop CC2015 where gene signals are isolated and background that could interfere with the script analysis was removed. Script processing was done using IP Labs software and Croft et al script (Croft *et al.*, 1999), whereby the nuclei were divided into 5 concentric rings of equal area and the intensity of gene signal (green) and the intensity of chromatin signal (blue) are measured. When the gene signals were normalised against the DAPI accurate extrapolation of gene positioning is achieved comparable to that using 3D methods.

3.2.4 Gene Expression Analysis

RNA was extracted from the remaining tissue of the snails after ovotestis had been removed. This was accomplished using either TRI-Reagent or RNAzol RT (Sigma, UK). In both cases samples were first macerated in solution using an axygen tissue grinder and then frozen at -80°C until used.

TRI-Reagent Protocol

Samples were defrosted and spun at 12,000g for 10min at 4°C and the supernatants transferred to new tubes to remove excessive insoluble material. Samples were incubated at RT for 5min before the addition of 0.1mL 1-bromo-3-chloropropane and vortexing for 15s and a further RT incubation for 15min. The solutions were spun for 15min at 12,000g at 4°C. The top aqueous phase was removed from each tube and placed in a fresh microcentrifuge tube and 0.5mL of 2-propanol was added and mixed by inversion before incubating at RT for 10min. Samples were then centrifuged at 12,000g for 10min at 4°C. Supernatants were discarded and the remaining pellet was washed in 1mL 75% ethanol spun at 12,000g for 5min at 4°C and the supernatant discarded again. Samples were washed in 0.4mL of 75% ethanol and spun at 12,000g for 5min at 4°C the supernatant discarded, the sample spun done briefly and the remaining solution pipetted out and the sample allowed to air dry for 15-30s and then dissolved in 20µL RNase Free water initially.

RNAzol RT Protocol

Samples were defrosted and spun at 12,000g for 10min and supernatants transferred to new tubes to remove any excessive insoluble material. Samples were incubated at RT for 10min followed by addition of 0.4mL of RNase free water to the samples to precipitate the DNA and proteins, samples were mixed via inversion and further incubated at RT for 15min. Following incubation the samples were spun at 12,000g for 15min at RT. Supernatants were carefully pipetted out of the tube into a new microcentrifuge tubes keeping track of total volume of each sample. Once transferred isopropanol was added to each sample to form a 1:1 ratio of supernatant to isopropanol precipitating the RNA. The samples were mixed via inversion and incubated at RT for 15min after which they were spun at 12,000g for 8min. Supernatants were discarded and the pellets washed in 0.4mL of 75% ethanol twice, centrifuging at 4,000g for 5min between each wash and discarding the supernatants. After the final wash the samples were spun down again, the remaining supernatant was carefully removed via pipetting. The pellet was allowed to air dry for 15-30s and then dissolved in 20µL RNase Free water initially

1µL of each sample was then further diluted in 9µL of RNase Free water and analysed for quantity and quality on a thermo nanodrop 2000. Genomic DNA contamination was removed using DNAse 1 (AMPD1, Sigma-Aldrich,UK). 2µg of RNA from each sample was treated with 2µL (2U) of DNAse 1 and made up to 20µL total volume using 2µL of 10x Reaction buffer and RNase Free water. Samples were incubated at 37ºC for 6h, after which 2µL of stop solution was added followed by a 70ºC incubation for 10min and finally cooled to 4ºC. Samples were then split into two and underwent conversion into cDNA using the Superscript IV VILO kit (Thermo Fisher Scientific, UK) 4µL of either the Master Mix or no-RT Master Mix control, 11µL of DNAse 1 treated RNA and 5µL RNase free water and mixed via pipetting. Samples were spun down briefly and incubated at 25ºC for 10min, 50ºC for 10min and finally 85ºC for 5min to convert the RNA into cDNA. Samples from both the active reverse transcriptase and negative control were then run at a 1/10 dilution on a Quantstudio 7 (Applied Biosciences, UK) qPCR machine to assess the samples for gDNA contamination.

Once the samples were clear of gDNA contamination they were run in triplicate for *actin*, *ferritin* and *hsp70*, *myoglobin* was used as an internal control. The cycle used for amplification was 50ºC for 2min for UDG activation to remove RNA contamination of the sample, 95ºC for initial denaturation and then 40 cycles of 95ºC 15s, 58ºC for 1min, followed by a melt curve analysis as a final step.

The primers used were actin (F: 5'-GGAGGAGAGAGAACATGC-3'; R: 5'CACCAATCTGCTTGATGGAC-3), ferritin (F: 5'-CTCTCCCACACTGTACCTATC-3'; R: 5'-CGGTCTGCATCTCGTTTTC-3') and hsp70 (F: 5'AGGCGTCGACATTCAGGTCTA-3'; R: 5'-TGGTGATGTTGTTGGTTTTACCA-3') with myoglobin (F: 5'-GATGTTCGCCAATGTTCCC-3'; R: 5'AGCGATCAAGTTTCCCCAG-3') as the internal house keeping gene for normalisation. Analysis was performed by comparing the fold change in gene transcript using the $\Delta\Delta$Ct method and student T tests performed to assess if significant difference in expression were achieved between BDM treated and control infected *B. glabrata*

3.2.5 Pilot Drug Treatment and Analysis

10mM of 2,3-Butanedione monoxime (BDM) was prepared using sterile water and juvenile snails were treated individually for 15min by placing them into a well of a 24-well plate containing 1mL of the 10mM BDM solution. After drug treatment snails were removed from the solution and washed twice using lepple aquarium water before being exposed to the

parasite as per 2.2.4. Initial analysis of BDM effectiveness as a treatment was performed using two methods, overall resistance whereby the number of snails that exhibited resistance to infection were counted each week and via shedding counts. Shedding was induced in the snails once a week by covering their tanks in foil to block out the light for at least 12h prior to shedding and then placing individual snails into a well of a 24-well plate containing a known volume of lepple water, enough to cover the snail, and then placing them under a direct light source. This induced shedding of parasites from the snail into the water. After 1h the light source was removed and the snails washed and replaced in their tanks. A 1/10th volume of Lugol solution (Sigma Aldrich, UK) was then added to each well to stain and neutralise the parasite. Using a dissecting microscope individual wells were inspected to see if any parasites were present in the wells to assess resistance to infection. Wells that were positive for parasites had shedding counts performed on them, this involved taking 12 aliquots of 5µL from each positive well, counting the number of cercariae in each aliquot and then averaging it out and calculating the number of cercariae for the known volume of solution.

3.2.6 BDM Dose Optimisation

20 snails were taken for each group and exposed to BDM as previously outlined in 3.2.5. Snails were exposed to varying concentrations of the drug as follows, 20mM, 50mM, 0.1M, 0.2M, 0.5M and 1M. After exposure the survival of each snail group was tracked over the following week. A separate group was given 20mM dose for 15min daily to assess how they would respond to a multi-dose trial.

3.2.7 Drug Assay Trials

The same methodology as outlined in 3.2.5 was used for later experiments for 0.1M BDM, 1µM methylene blue (Sigma Aldrich, UK), 1µM remodelin, 1µM anacardic acid (Sigma Aldrich, UK) and 2.5µM sodium butyrate (Sigma Aldrich, UK) with minor variations. Methylene blue and remodelin were added directly to the tanks water and snails were maintained in static tanks for 24h prior to infection. Anacardic acid and sodium butyrate snails were dosed in 35mL of sterile water in a falcon tube containing 15 juvenile snails for

48h, the falcon tubes were sealed with parafilm with air holes punctured in so they could not escape. Prior to infecting snails were washed twice and infected as outlined in 2.2.4.

3.3 Results

The host-pathogen relationship is a complex interplay of host immune response and pathogens immune evasion. With *S. mansoni* infection of *B. glabrata* one of the many factor involved in host compatibility to infection is *S. mansoni* ability to induce changes within the host nuclei to cause upregulation of target genes such as *hsp70*. Within is investigated the importance of non-random gene relocation to host gene regulation using a myosin inhibitor to prevent gene movement and the relationship between changes to the host acetylome via HATi and HDACi which would inhibit certain alterations to the host epigenome. Both of these factors, changes to the epigenome and inhibition of nuclear motors, could potentially affect the complex host-pathogen relationship and impact on host susceptibility to infection. By gaining a deeper understanding of how these factors contribute to susceptibility to infection new targets for intervention may become apparent.

3.3.1 2,3-Butanedione Monoxime (BDM) gp snail strain drug assay

Previous research (Arican-Goktas *et al.*, 2014) used two snail strains, the resistant BS90 and Susceptible NMRI to establish the gene movement induced by the parasite *Schistosoma mansoni* and showed the inducement of movement of the *hsp70* loci. This led to the establishment of a heat shock model of gene movement using the susceptible BB02 strain of snail. This snail however showed different localisation of *hsp70* gene loci to that established in the previous snails. The resistant BS90 had an intermediate gene position that did not move, NMRI had an intermediate gene position that moved to an internal position and in the heat shock model BB02 had an internal gene position that moved to an intermediate position after heat shock. To establish gene localisation in interphase nuclei an erosion script (Croft *et al.*, 1999) was used [Fig 3.1]. To establish if *S. mansoni* induced gene movement was essential to infection two areas needed to be investigated. First does *S. mansoni* induce gene movement in multiple strains of snail. Previous work by Arican-Goktas et all had investigated the NMRI susceptible lab strain so the gene pool (gp) lab strain and BB02 wild type susceptible strains were both investigated to establish that *S. mansoni* induced gene

movement in other susceptible strains. Once this was established the focus of the investigation was on the importance of gene movement to infection, this was explored using a global myosin inhibitor (BDM) known to affect nuclear myosins and shown to prevent heat shock induced gene movement in the BB02 strain (Arican-Goktas, 2013). This would elucidate whether preventing gene movement would increase the host snails resistance to infection, as gene movement did not occur in the resistant BS90 snail strain, and impact on expression of the target gene, *hsp70*.

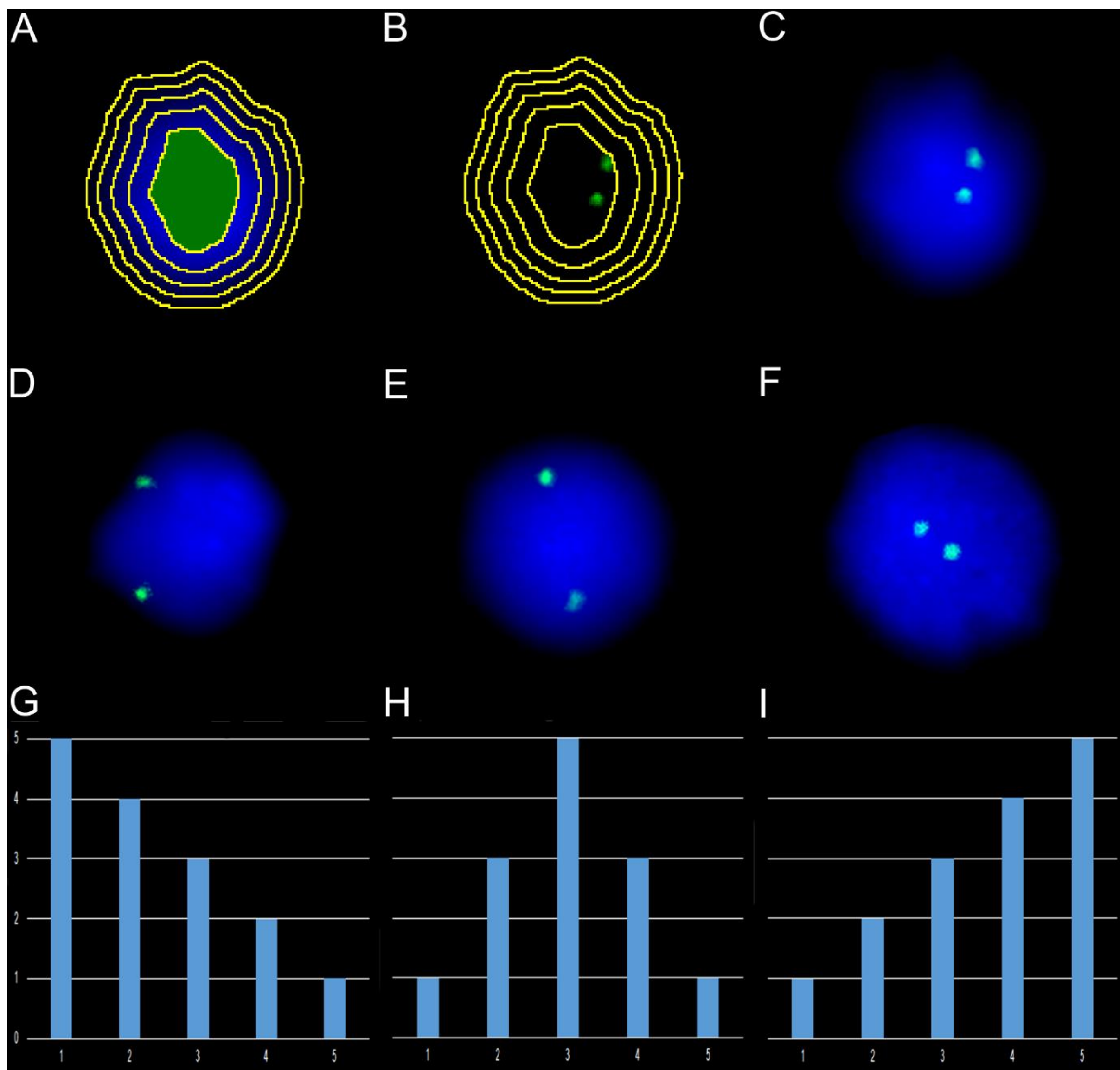


Fig 3.1 Representative images of the erosion analysis (A) Demonstrates the function of the script which divides nuclei into 5 concentric rings of equal area and then measures the intensity of the DAPI signal. (B) Demonstrates the constraint of FISH signal measurement to within the same concentric rings as the DAPI signal. FISH signal can be normalised by

dividing it by DAPI to take into account the relative density of DNA in different parts of the nucleus to extrapolate back into 3D positioning. (C) The image used for analysis shown in A and B. The 5 concentric rings created by the erosion script are referred to as shells with shell 1 being the most peripheral and shell 5 the most internal. Using this method FISH signal can be localised to one of three nuclear areas, periphery, intermediate or internal. D) Is a peripheral FISH signal, E) is an intermediate FISH signal and F) is an internal FISH signal. G) Is a representation of a graph generated to show peripheral FISH signal localisation, H) a graph that is representative of an intermediate FISH signal localisation and I) is a graph representative of an internal FISH signal localisation.

All infection work was performed at our collaborators laboratory at the Wellcome Trust Sanger Institute where they maintain the susceptible gene pool (gp) strain a third separate *B. glabrata* strain that was produced by cross-breeding several strains to create one that was optimised for susceptibility to the *S. mansoni* parasite for maintenance of its life cycle. The initial experiments conducted used this strain of snail and a global myosin inhibitor 2,3-butanedione monoxime (BDM) which had been shown to prevent gene movement after 1h heat shock in the BB02 strain (Arican-Goktas and Bridger unpublished data).

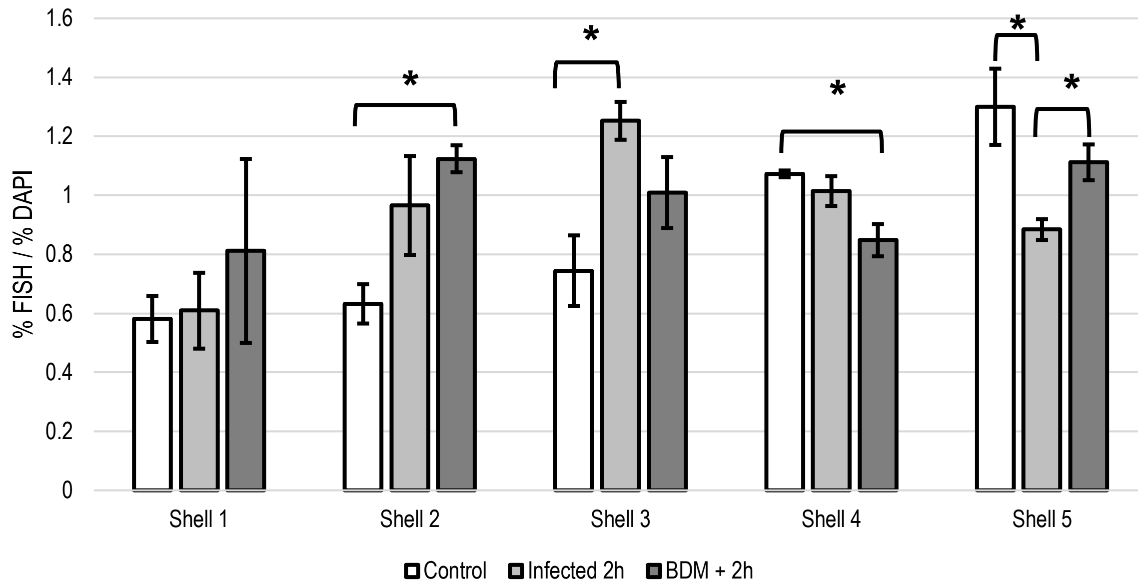


Fig 3.2 Relative gene position found in the gp strain in three different experimental conditions. Control, uninfected snails, Infected 2h, control snails 2h post infection and BDM + 2h, snails treated with 10mM BDM for 15min prior to infection 2h after infection. * indicates bars that are significantly different with p-value = < 0.05, error bars = S.E.M. n ≥ 50 nuclei, in triplicate

It was established that in the gp strain the normal gene loci positions are internal, same as that exhibited in the BB02 strain and upon infection it is moved to an intermediate position as demonstrated in Fig 3.2. This move to an intermediate position is the same not only as the heat shocked BB02 snails but also shows movement of the *hsp70* gene loci similar to that NMRI strain following infection, although going in the opposite direction. The BDM treated snails showed changes in gene position with an increase in intermediate signals but, does not show a significant decrease in internal signals. The BDM treated snails also exhibits significant differences from the control infected snails having more internally located gene loci. This may indicate that not all gene movement was halted resulting in a gene positioning pattern somewhere between the two controls with increased intermediate signals over uninfected controls but more internally located gene loci than controls two hours after infection. The remaining tissue, head-foot and hepatopancreas, from the subjects used to make the cell suspensions for FISH were processed for RT-qPCR to assess how *hsp70*, *actin* and *ferritin* gene expression was affected during infection and by BDM treatment.

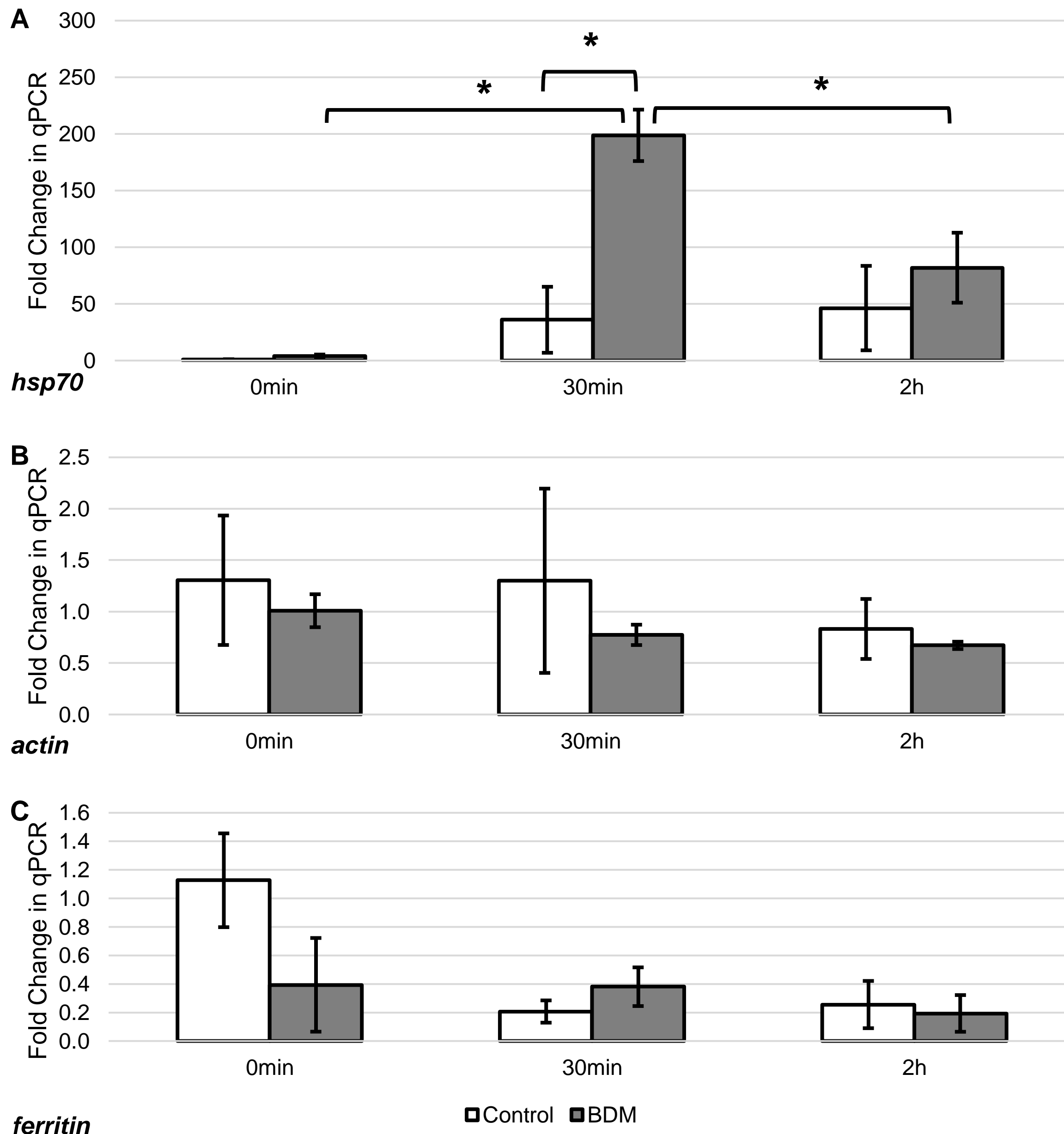


Fig 3.3 Comparative gene expression in gp strain snails when using the 0min Control as the baseline comparison for ΔΔCT calculations for A) *hsp70* gene expression which shows increases in *hsp70* gene expression in the BDM treated snails in comparison to controls, B) *actin* which shows potentially higher expression in the control when compared to the BDM treated and C) *ferritin* which exhibits no differences between BDM treated and controls. * indicates bars that are significantly different with p-value = < 0.05, error bars = S.E.M

Previous work, (Arican-Goktas *et al.*, 2014), has shown that both *actin* and *hsp70* gene expression is upregulated in NMRI strain *B. glabrata* after infection by *S. mansoni* parasites. In the gp strain there is a definite increase in expression of *hsp70* shown in both the treated

and untreated infected samples although time for expression peaks seem to differ with the BDM peaking at 30min and the control showing increasing expression continuing on until the 2h time point relative to their constitutive controls as displayed in Fig 3.3A. However no increase in gene expression was evident from either the *actin* or *ferritin* genes as is evident in Fig 3.3B and 3.3C. The previous study (Arican-Goktas *et al.*, 2014) did show increases in *actin* expression but, this could be a strain specific event.

When BDM treated samples were compared to the internal BDM treated 0min control the gene expression fold increases demonstrated a similar pattern expression as the control group. However as can be seen in Fig 3.3 when comparing to the untreated 0min control you can see a significant change in gene expression profile of the *hsp70* gene, as demonstrated in Fig 3.3A, which is likely due to a combination of both the parasite and the BDM treatment resulting in increased *hsp70* gene expression. *Actin* and *ferritin* gene expression still exhibited no significant changes between treated and untreated snails post infection as exhibited in Fig 3.3B and Fig 3.3C respectively. To assess the overall difference that results from the BDM treatment alone, control samples were compared to BDM treated samples at each time point [Fig 3.4].

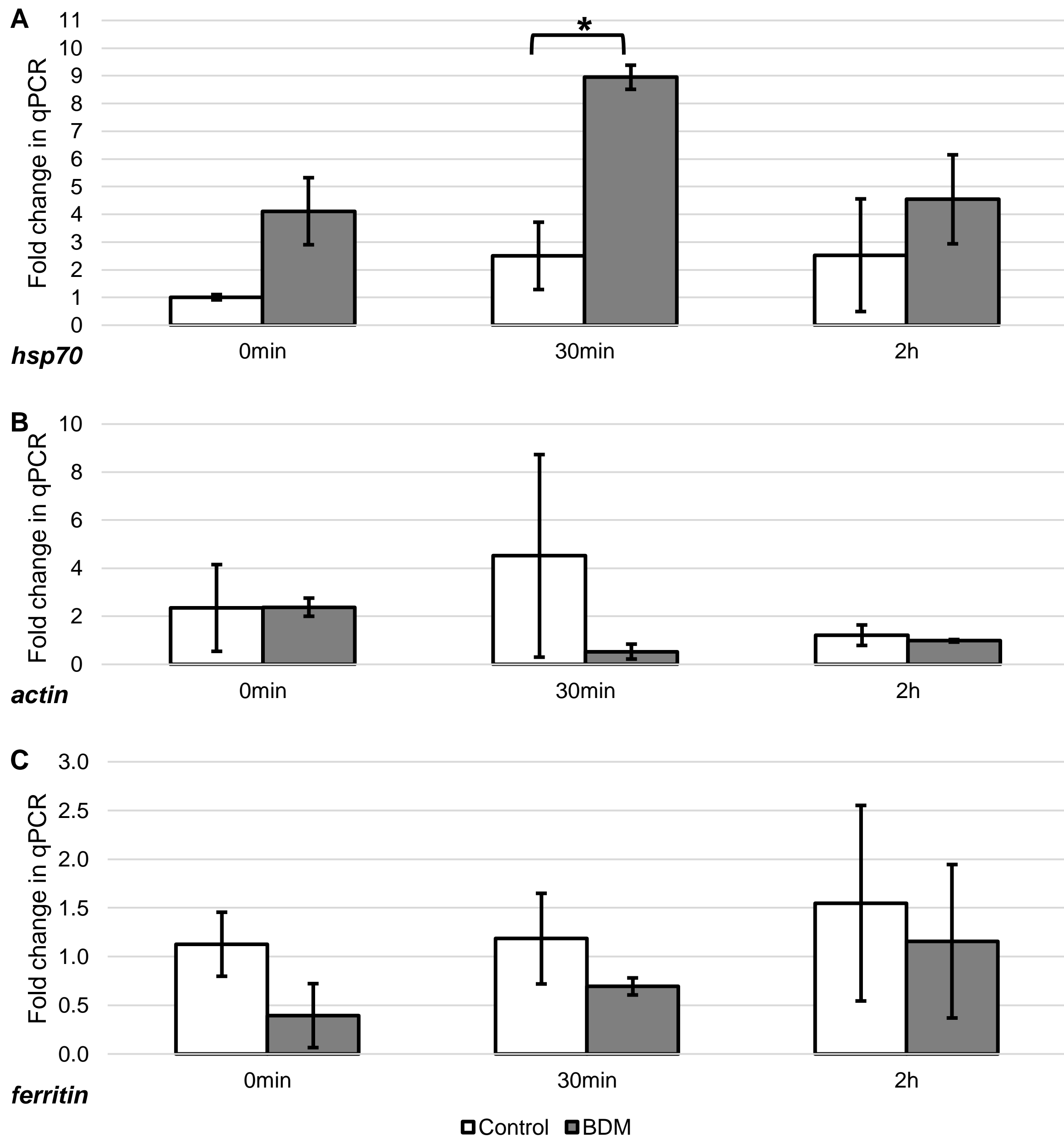


Fig 3.4 Increases in gene fold expression in gp strain snails when comparing Control to BDM treated ΔΔ-CT to each other at time point. Assessing the effect of BDM treatment while controlling for *S. mansoni* infection. A) *hsp70* gene expression is consistently elevated due to BDM treatment when infection is controlled for, B) *actin* gene expression and C) *ferritin* gene expression both show comparatively little change as a result of BDM treatment when infection is controlled for, although in both cases BDM treated does appear to cause a reduction in *actin* and *ferritin* gene expression in comparison to controls. * indicates bars that are significantly different with p-value = < 0.05, error bars = S.E.M

Fig 3.4 displayed that BDM treatment alone does seem to elicit changes in gene expression on its own as well. In particular *hsp70*, as demonstrated in Fig 3.4A, which was the main target and it was hypothesised that preventing gene movement may prevent upregulation of the *hsp70* gene yet the BDM itself causes a spike in *hsp70* gene transcription. Because of this it will be difficult to assess whether preventing gene movement did in fact prevent the parasite from further enhancing *hsp70* expression and whether preventing gene movement could be a viable target for preventing infection. Following the FISH and qPCR results the long term effects of BDM treatment were examined by looking at the susceptibility to infection [Fig 3.5] and the overall cercariae shed from the snails [Fig 3.6]

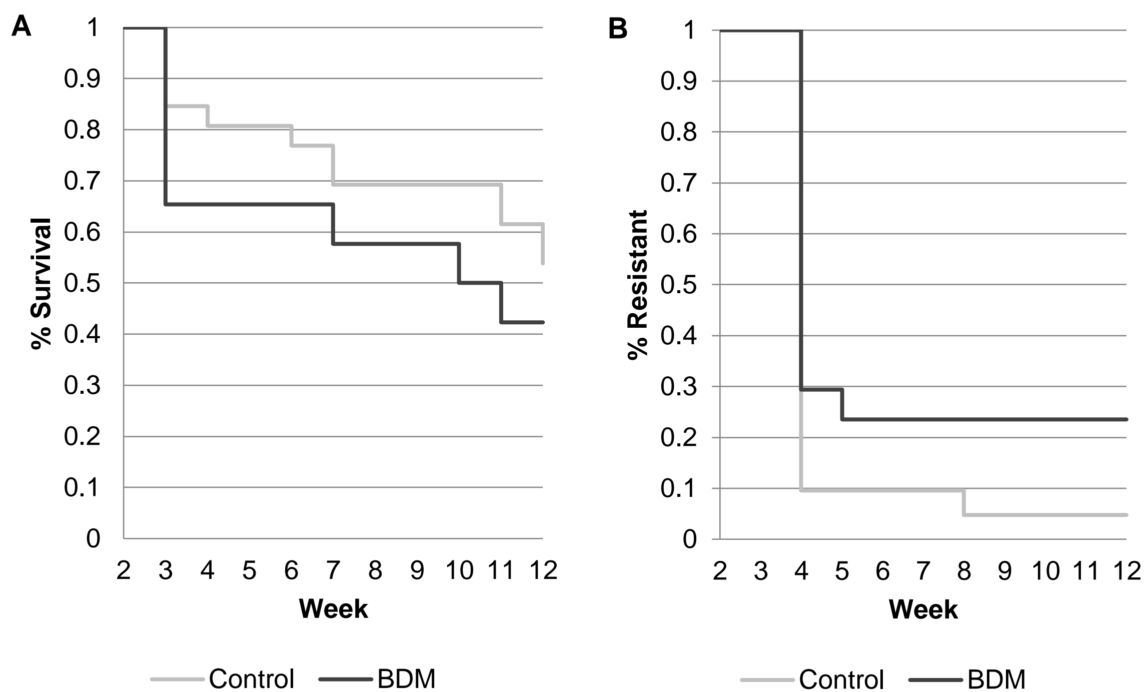


Fig 3.5 Kaplan-Meier graphs comparing Control (n= 24 snails) to BDM treated (n= 24 snails) snails over a period of 12 weeks. A) The survival of the two experimental snail groups, Control and BDM treated. B) The overall resistance to infection exhibited by the two experimental groups, Control and BDM treated. The BDM treated group exhibit no statistically significant change in either survival or resistance when compared to the control group using a Log-Rank test.

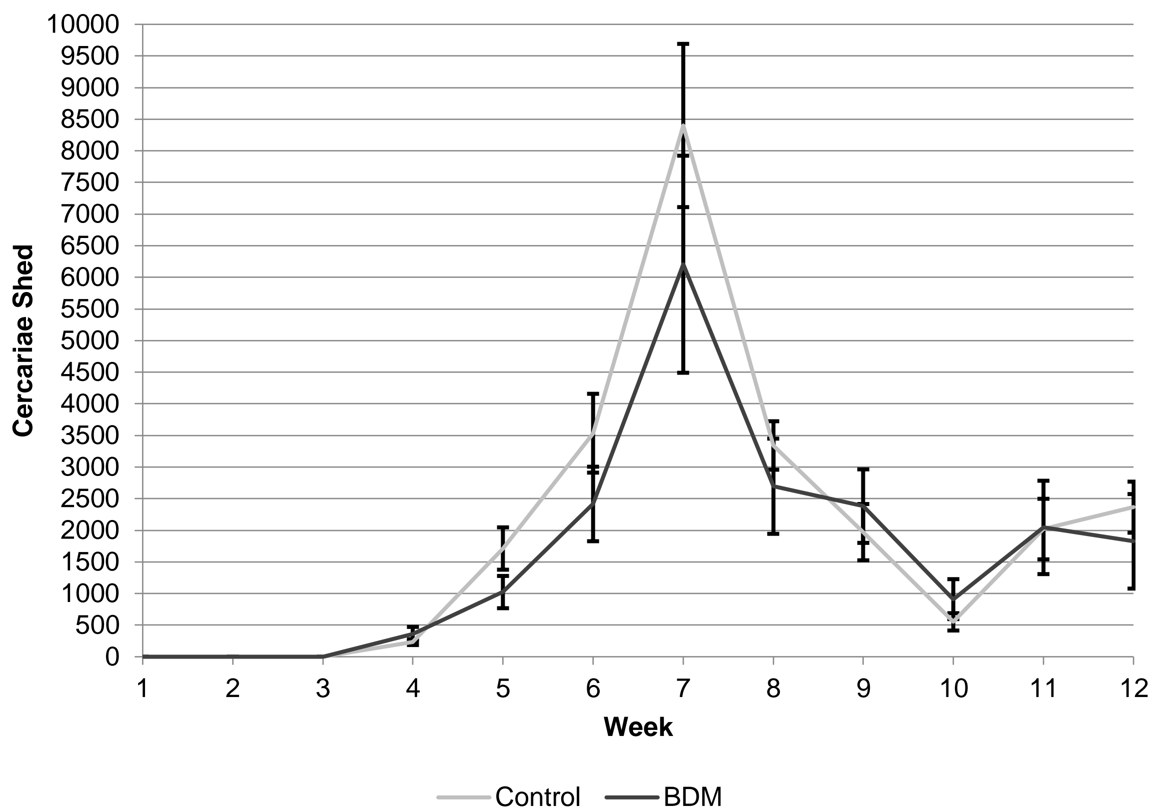


Fig 3.6 Average cercariae shed from each experimental group, Control (n= 24 snails) and BDM treated (n= 24 snails), over the 12 weeks of the experiment, error bars = S.E.M. Counts involved taking the average number of cercariae from 12 aliquots containing 5µL of the water a snail was shed in and calculating the number of cercariae by volume.

The FISH data shown in Fig 3.2 does show that BDM treatment has an effect on gene movement and the overall resistance to infection in the BDM treated group was four fold greater in raw numbers, although this was not statistically significant. It also showed that BDM on average did generally shed less however, this decrease in average cercariae shed may be attributable to the greater number of resistant snails in the treated group lowering the average shed per snail for the group. This would seem to indicate that the amount of cercariae shed by a snail is not being affected by the BDM treatment. This pilot test indicates that there was potentially an increase in resistance offered by the BDM but further experimentation was warranted.

The concentration of the BDM dose used in the experiment was to keep it in line with the previous heat shock experiments but no upper limit was established. Before rerunning the

BDM experiment a maximum survivable dose was investigated to maximise the effects treatment may have on the snails. The results of which are shown in Fig 3.8.

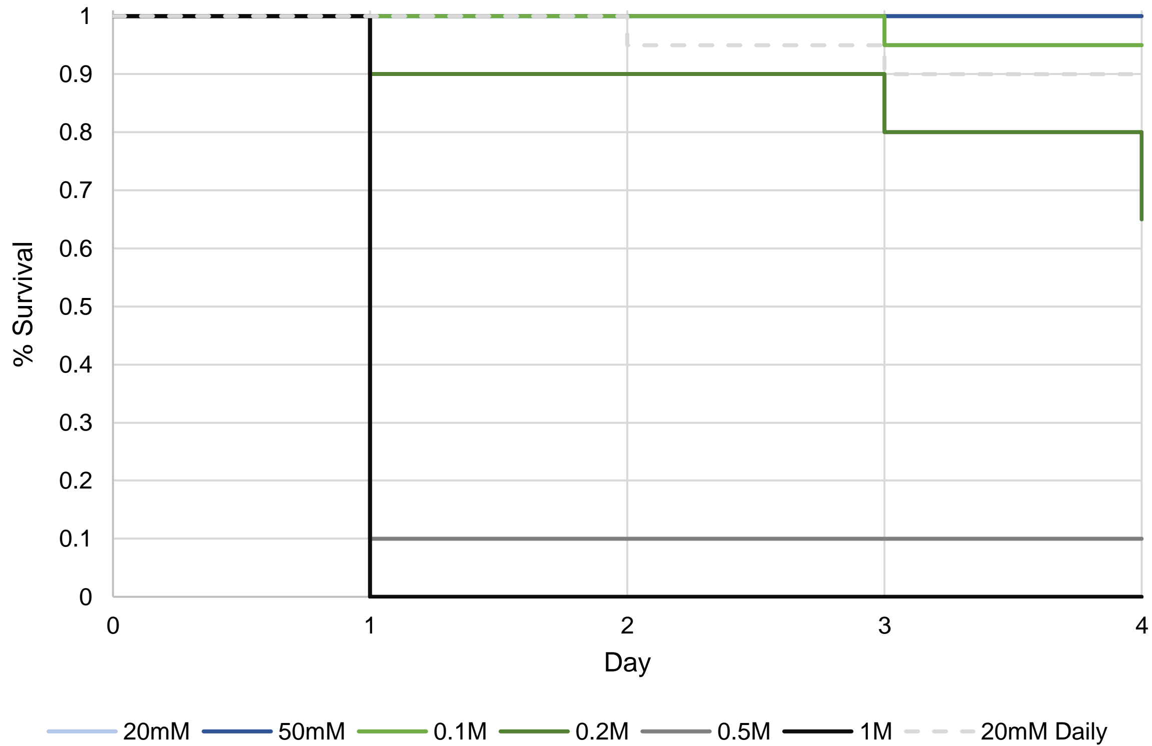


Fig 3.7 Kaplan-Meier graph showing the overall survival of snails over 4 days for treatment using different concentrations of BDM (n= 20 snails per dose). 20mM and 50mM concentration were both tolerated without any deaths. 0.1M displayed a minor decrease in tolerability with higher concentrations quickly exhibiting lethality. The 20mM multi-dose was more tolerable than a single 0.2M dose but less so than a single 0.1M dose though it did demonstrate than a multi-dosing protocol may be possible.

The dosing experiment demonstrated that the LC50 for the snail was likely between 0.2M and 0.5M, however, survival post infection was also highly variable so to ensure the maximum number of snails survived it was prudent to use a lesser dose where survival was near 100%. For this reason 0.1M was chosen for future experiments, it was an order of magnitude greater than the previous dose used and the only death in the group was what appeared to be the weakest member of the cohort.

3.3.2 BDM BB02 snail strain drug assay

A larger cohort drug study was conducted using the optimised BDM dose. The Wellcome Sanger Institute snail facilities although adequate for infection and maintenance of a life cycle was not capable of breeding the numbers required so instead we used snails bred and maintained at Brunel University London, the BB02 strain. These would be transferred to the facilities at the Sanger Institute two days prior to infection. Alongside the BDM two other drugs would also be tested, the HSP70 inhibitor methylene blue (MB) and NAT10 inhibitor remodelin (R), both at 1µM concentrations for 24h prior to infection. No cercarial count was taken during these experiments and only resistance was measured.

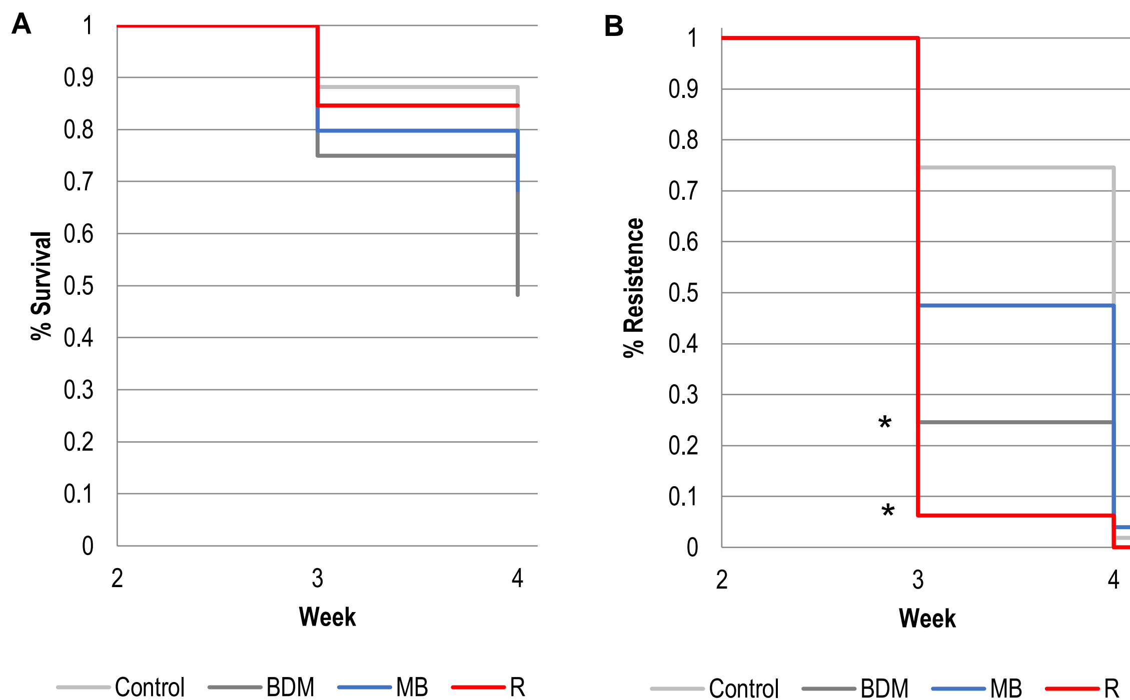


Fig 3.8 Kaplan-Meier graphs for comparison of the four groups Control (n= 76 snails), BDM (n= 76 snails), Methylene Blue (MB) (n= 74 snails) and Remodelin (R) (n= 78 snails) over the course of 4 weeks of experimentation A) The survival of the four experimental groups, Control, BDM, MB and R. B) The overall resistance to infection exhibited by the four experimental groups, Control, BDM, MB and R. All four groups showed comparatively better survival than that of the previous study however, both BDM and R showed significant reductions in resistance to infection. * indicates statistically significant changes using a Log-Rank test to compare two curves p-value = <0.05.

The second drug trial showed significantly different results from the first, although survivability was not much different between the groups there was significant difference in resistance seen in both the BDM and remodelin groups with both showing significantly earlier onset of shedding and even the MB group demonstrated some earlier shedding than the control group. For BDM this contradicted what had been previously shown in the smaller pilot study. Similarly the *hsp70* gene loci positioning was now showing that the BDM had had some effect on gene positioning as with the initial trial with it neither matching the control 0min or the control 2h but seeming to occupy a midpoint between the two. The BDM treated cohort exhibited an intermediate position predominantly in shell 4 rather than shell 3 and shell 2 as with the control infected while still showing a shift from the internal shell 5 position that uninfected control cohorts possessed [Fig 3.9]. This was not too surprising as the initial study had shown that the BDM+2h infection group also exhibited a different gene positioning [Fig 3.2] from both control groups although canonical internal positioning was not significantly affected in the first trial in the BDM+2h infection group.

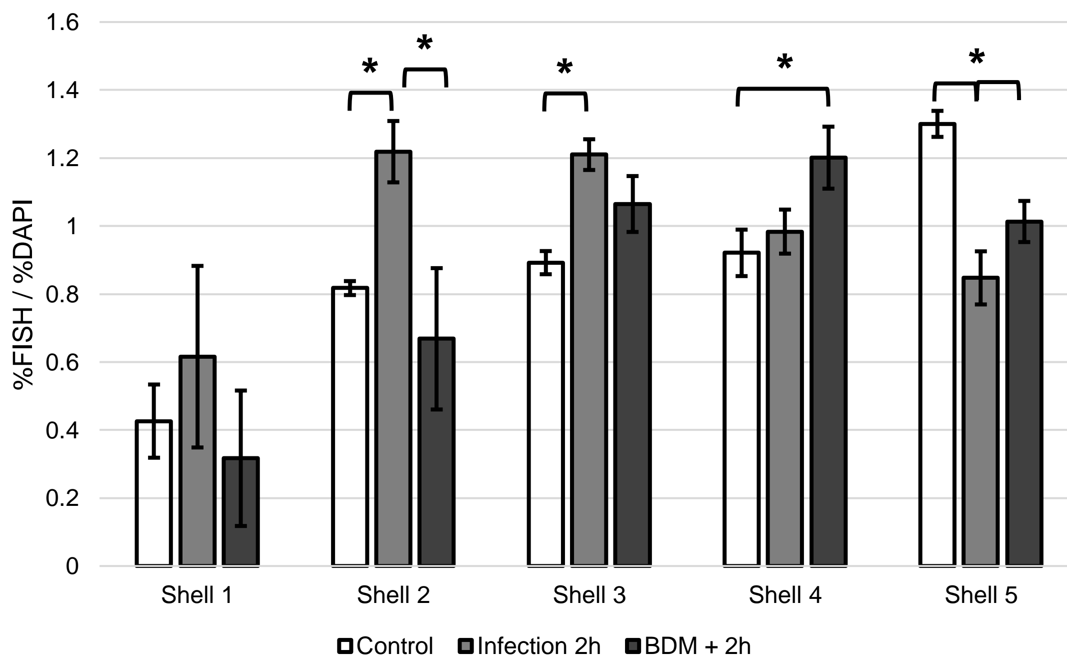


Fig 3.9 Relative gene position found in the BB02 strain in three different situations. A) Control, uninfected snails, B) Control snails 2h post infection, C) 0.1M BDM treated snails 2h after infection. * indicates bars that are significantly different p-value = <0.05, error bars = S.E.M. n ≥ 50 nuclei, in triplicate

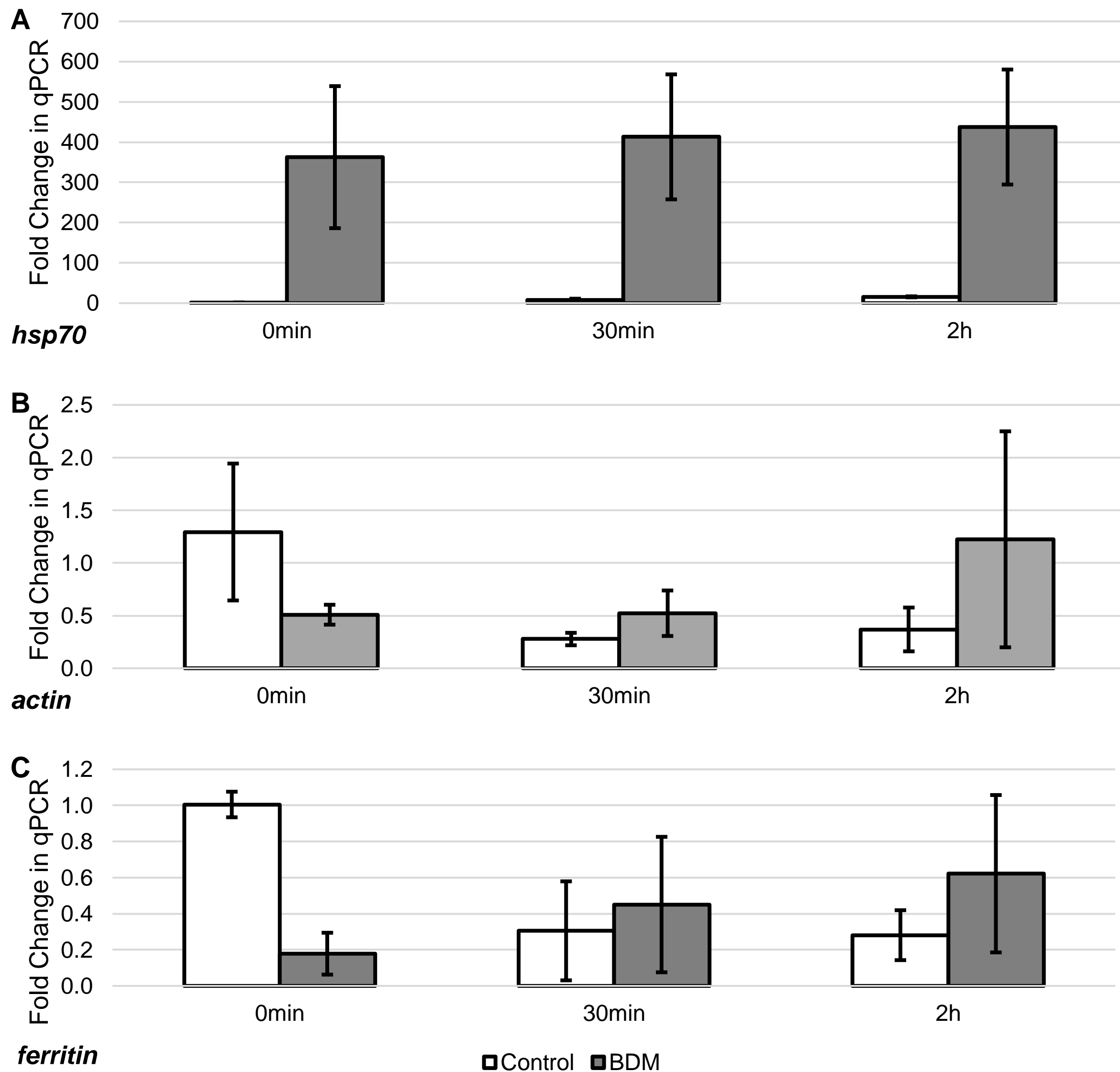


Fig 3.10 Comparative gene expression in BB02 strain snails when using the 0min Control as the baseline comparison for ΔΔCT calculations for A) *hsp70* gene expression which shows increases in *hsp70* gene expression in the BDM treated snails in comparison to controls, B) *actin* gene expression C) *ferritin* gene expression. B and C once again show a similar pattern with initial expression of both *actin* and *ferritin* being lower in the BDM cohort but with it increasing as the control cohort decreases at 30min so expression is higher in BDM cohort at both 30min and 2h post infection.

In contrast to what was previously seen during the gp strain experiments BDM treatment initially seemed to stop any increase in *hsp70* gene expression. In contrast when comparing 0min controls to 30min and 2h time points an increase in gene expression was obsereved. *Actin* gene expression similar to the gp strain remains relatively static, which differs from that

seen during experiments with the NMRI strain where *actin* expression increases at the 2h time point. This could be a strain specific response or the activation of the actin upregulation may come later in the BB02 and gp strains. There is also upregulation of *ferritin* within the BDM treated samples. However, since *ferritin* upregulation was linked to physical damage response with the NMRI strain using irradiated miracidia (Arican-Goktas *et al.*, 2014) it is likely this is a response to the higher concentration of BDM used. Greater concentration of BDM caused osmotic injuries in snails and potentially even at this safe dose it could cause some injury to surface cells during exposure and the increase in *ferritin* expression seen here could be in response to such injury.

However, when comparing all samples to the 0min untreated control [Fig 3.10] the combination of being BDM treated and infected significantly increased gene expression of *hsp70* as is demonstrated in Fig 3.10A. In fact in this case the effect is so dramatic, that there seemed to be no increase in expression in the BDM treated samples as a result of *S. mansoni* infection. This could be due to the significantly increased dose, 0.1M compared to 10mM, or strain differences between the gp and BB02 strains or a combination of these factors. However, in comparison to untreated 0min controls a significant increase in gene expression is readily apparent. In contrast neither *actin* or *ferritin* gene expression is significantly altered as is exhibited in Fig 3.10B and Fig 3.10C respectively. When controlling for time and infection it is evident that BDM treatment causes significant increases in *hsp70* expression as had been previously shown in Fig 3.4. In this case it would appear that at 0.1M concentration in the BB02 strain the *S. mansoni* parasite could not elicit any further increase in gene expression than was already occurring due to the BDM treatment.

The data so far from the two experiments have been consistent with one another despite being derived from two different strains of the snail except for the long term effects on snail resistance to infection. To get a more solid conclusion on the effects on long term resistance a third trial for BDM was conducted, still using the BB02 strain and the 0.1M concentration to assess the effects on resistance.

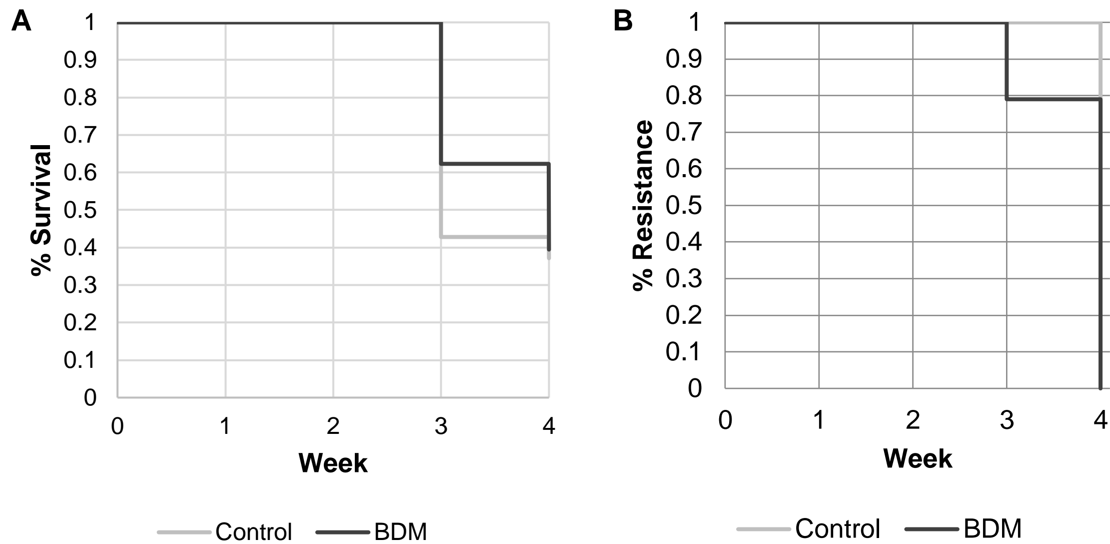


Fig 3.11 Kaplan-Meier graphs for comparison of the two groups, Control (n= 70 snails) and BDM treated (n= 61 snails), over the course of 4 weeks of experimentation. A) The survival of the two experimental groups, Control and BDM treated. B) The overall resistance to infection exhibited by the two experimental groups, Control and BDM treated. Log-Rank tests for comparing two curves showed no statistically significant change between the two cohorts.

The third BDM trial showed no significant difference in survival [Fig 3.11A] or resistance to infection between control and treated groups, either positive or negative [Fig 3.11B]. However, the same conclusion cannot be said about preventing gene movement since the BDM did not prevent the parasite induced gene movement in either of the first two studies although it did have some effect on gene position in the gp strain. Similarly the gene of interest in this experiment was *hsp70*, due to the ease of using a heat shock model for gene movement and its apparent import in infection, however, although the gene may or may not have moved it is evident that BDM treatment also induces upregulation of *hsp70* maybe through another mechanism than that of the parasite meaning that to identify whether preventing gene movement prevented upregulation and infection a method which did not cause up regulation of the target gene would also need to be identified.

3.3.3 The effects of inhibiting histone acetyl marker changes on infection

During the large cohort study alongside BDM two other drugs were investigated, one of which showed significant effects on snail resistance. Remodelin is a NAT10 inhibitor that is known to prevent acetylation of several proteins including histones. During the previous experiment it showed significantly earlier shedding than the control group and although not quantified the number of parasites shed appeared to be significantly greater than the control. To confirm this a smaller experiment was conducted where cercarial counts were taken the results of which are shown in Fig 3.12.

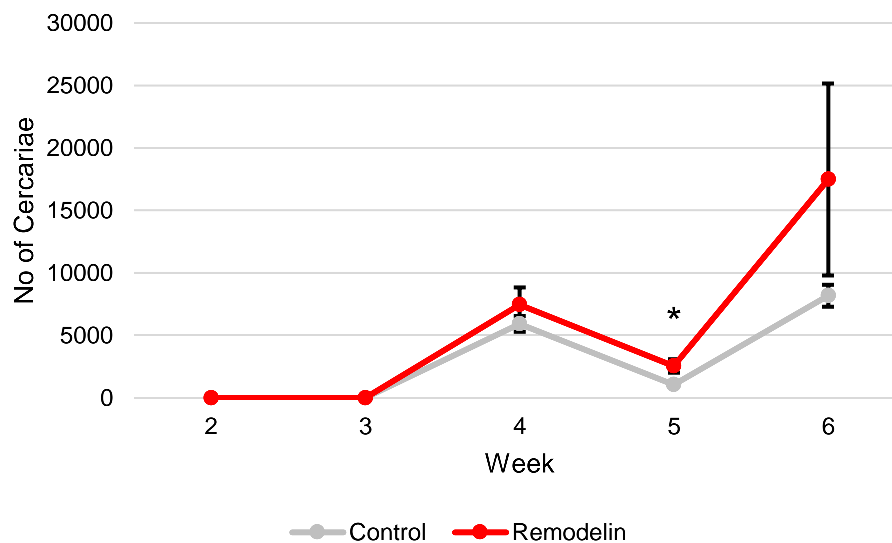


Fig 3.12 Average cercariae shed from the snails of either the Control or Remodelin treated cohorts. This shows that Remodelin treated snails consistently shed more on average than Control snails. * indicates statistically significant differences as derived from a Two-Tailed, equal variances, Student T-Test with p-value = <0.05, error bars are = S.E.M

This trial did not replicate the earlier shedding point seen previously however it did show consistently that the remodelin treated snails were shedding more parasites on average. This raised the question of whether it was remodelin specifically or if the manipulation of acetyl markers on histones in general would have the same effect. To this end two drugs were trialled, anacardic acid (AA) a HAT inhibitor and sodium butyrate (SB) a HDAC inhibitor, the former preventing the addition of acetyl marks as remodelin would have done and the latter preventing the removal of acetyl markers. Both were tested for toxicity and

snails had 100% survival after being kept in active concentrations of the drugs for 4 days so no toxicity issues were apparent and snails would be dosed for 48h prior to infection.

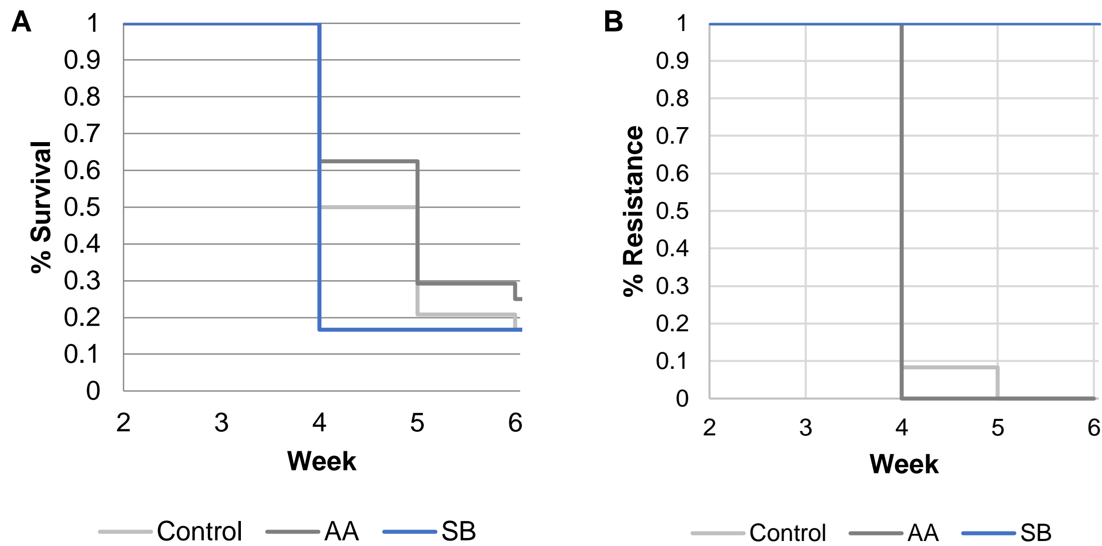


Fig 3.13 Kaplan-Meier graphs showing comparisons between the three groups, Control (n= 24 snails), Anacardic Acid (AA) (n= 24 snails) and Sodium Butyrate (SB) (n= 12 snails) over a 6 week period post infection. A) The survival of the three experimental groups, Control, AA and SB. B) The overall resistance to infection exhibited by the three experimental groups, Control, AA and SB. Log-Rank tests were performed for statistical analysis and no statistically significant different was found when comparing either of the treated cohorts to the controls.

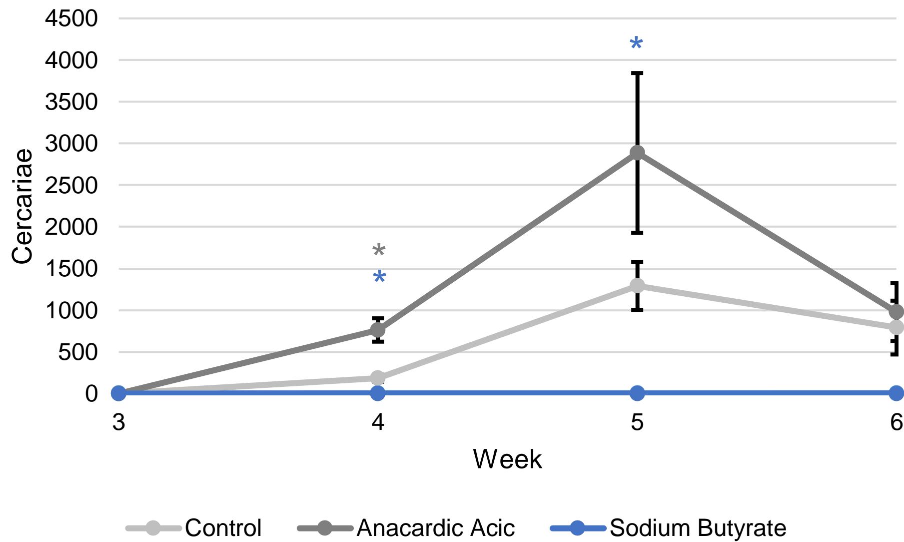


Fig 3.14 The average number of cercariae shed from each group over the 6 weeks of experimentation. The treated groups were compared to the controls at each time point using a Two-Tailed, unequal variances, Student T-Test with a p-value = <0.05, * indicates significant changes from the control cohort. Error bars = S.E.M

This first experiment with AA and SB showed that the HAT inhibitor AA mimicked what had the most recent remodelin trial as the number of shed cercariae parasites were significantly increased early on after infection and consistently shed more cercariae on average although significance was lost after the first week. Sodium butyrate the HDAC inhibitor displayed the complete opposite with 100% resistance exhibited in all surviving test subjects which went on beyond the weeks shown until the 9th week when the experiment was ended for the SB group as week 8 is the last point at which shedding could potentially occur in an infected snail. However due to a decreased survival rate in the experimental groups a final experiment was conducted assessing remodelin, AA and SB.

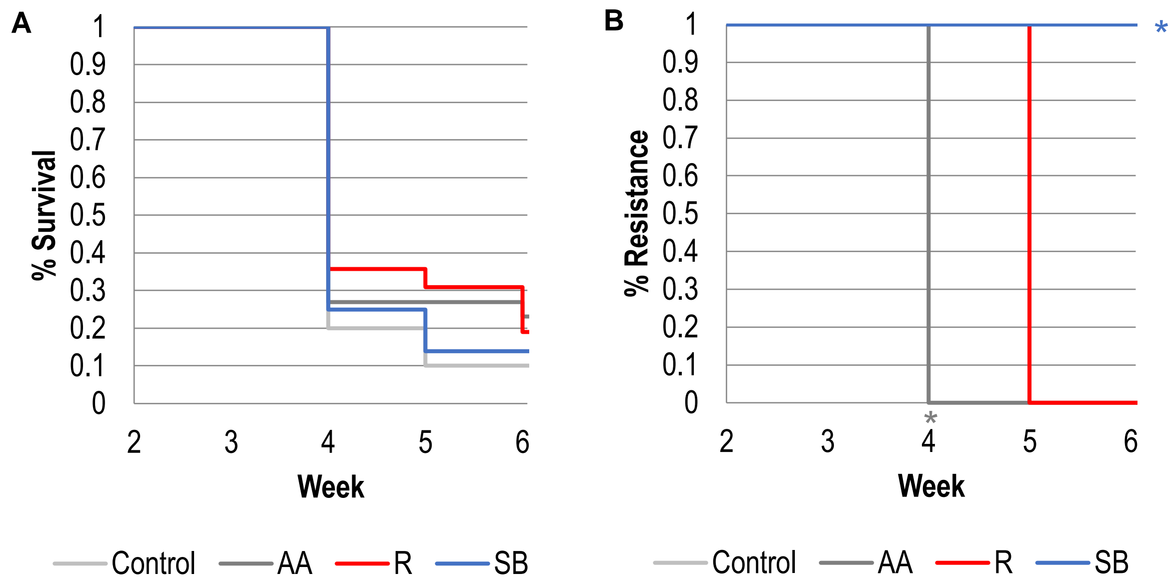


Fig 3.15 Kaplan-Meier graphs comparing four groups, Control (n= 40 snails), Anacardic Acid (AA) (n= 26 snails), Remodelin (R) (n= 42 snails) and Sodium Butyrate (SB) (n= 36 snails), over 6 weeks of experimentation. A) The survival of the four experimental groups, Control, AA, R and SB. B) The overall resistance to infection exhibited by the four experimental groups, Control, AA, R and SB. Log-Rank tests were conducted to compare treated cohorts to the controls, statistically significant curves are denoted by * with p-value = <0.05

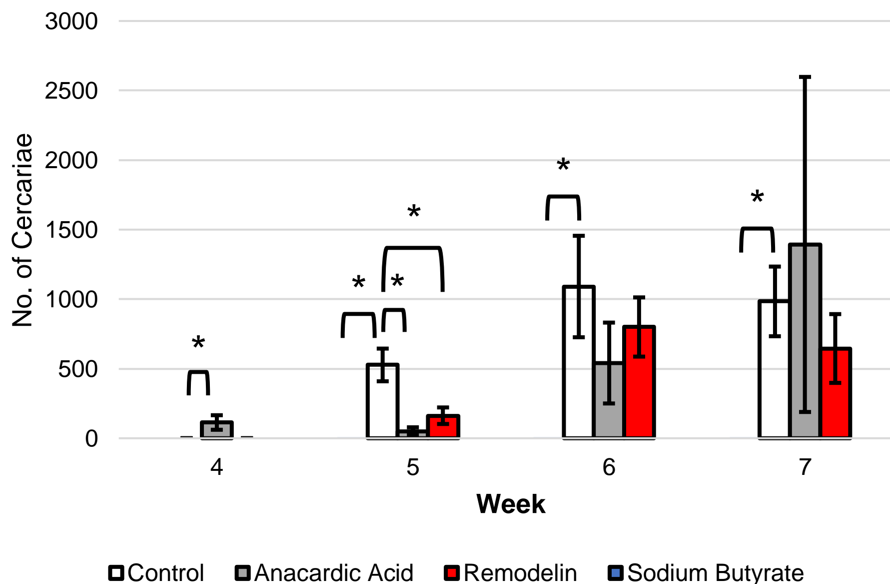


Fig 3.16 The average number of cercariae shed from each group over seven weeks. Two-Tailed Student T-Tests were used to compare each treated cohort to the controls, p-value = <0.05 * = statistically significant difference. Error bars = S.E.M

This time remodelin did not demonstrate any increased susceptibility in fact the numbers of cercariae shed from the remodelin group were significantly less at week 5 in comparison to control, the same was true for AA as well, however AA also shed a week earlier than all other groups at week 4 which holds to the pattern that HAT inhibitors may increase *B. glabrata* susceptibility to infection and by the end of the 7th week AA was once again shedding more than the control group. It would appear that HAT inhibition results in either earlier or greater average number of cercariae shedding which could be indicative of an increased speed of maturation of the parasite in the host. The theory of HAT inhibition increasing susceptibility is also supported by the action seen by its opposite, the HDAC inhibitor which once again afforded complete resistance to parasite infection. It would appear that preventing the addition of acetyl marks to histones increases a snail's susceptibility to infection while preventing their removal offers greater resistance to infection or may even block infection entirely.

3.4 Discussion

Schistosomiasis is a debilitating tropical disease for which there is currently only one universal treatment which does not affect immature adult parasites and requires at least two doses to ensure clearance of the parasites (Cioli, 2014). In order to develop new effective treatments or discover potential targets we need to have a clearer understanding of how the parasite infects its host and what interrupts it. Initially the focus was on preventing gene movement that was exhibited in a time response manner to upregulation of gene expression. The main focus was on *hsp70* as this gene loci could be moved in a heat shock model and movement was halted. This led to the current development of a drug assay model for snail infection that ideally would allow for the testing of new compounds with known targets to see how they affected susceptibility of *B. glabrata* to *S. mansoni* infection.

The initial target was nuclear myosin, a protein thought to be a component of the nuclear motor necessary for gene movement (Mehta, 2010). The drug used was 2, 3-Butanedione monoxime (BDM), this was not ideal as this is not specific for nuclear myosin but inhibits all myosins so would have a plethora of off target effects. Although it did prevent gene movement in the heat shock model it was unable to effectively prevent gene movement during infection. Furthermore, part of what was hypothesised to act as ameliorating factor

would be the reduction in *hsp70* expression that was shown to become upregulated following gene repositioning. However, the exposure to BDM also resulted in significant upregulation of *hsp70* gene expression in both the gp and BB02 strains which is a complicating factor in this case making it difficult to assess if preventing gene movement would prevent parasite induced upregulation and making it impossible to assess if reduction in *hsp70* expression would result in increased resistance to infection.

The BDM work however did highlight some issues that would need to be addressed in moving forward with the development of an assay for assessing the effects of a compound on resistance to infection. Firstly is assessing overall susceptibility to infection, although initially the pilot study used two measures, overall resistance and cercarial count the second trial only used one factor, overall resistance. This was because the pilot study had shown no significant difference in cercarial shedding, the loss of a second measure for control was highlighted however with the remodelin trial run concurrent to the second BDM trial. Not only did the snails on the remodelin treatment shed significantly earlier in that first trial it was obvious from the wells the snails were shed in that they were also shedding significantly more but, this was not accounted for at this time. Resistance when investigating a disease as complex as a parasite is not solely about being completely immune to infection as changes in the success of the parasite, such as an increased cercarial shed or changes in incubation times, could indicate potential mechanisms of interest. Significant changes in number of cercariae could indicate factors affecting their survivability that can then be investigated and refined to identify pathways and drugs that could have greater success due greater specificity if it was a result of an off target effect or improved binding efficiencies.

The second issue that came to light was survival of the snails. Generally young snails are used and in these experiments juveniles, ranging from 2-5mm in size, as at this stage they have not begun to sexually mature and have the greatest susceptibility. This however, resulted in issues of survival leading up to shedding with loses of 10-55% within the first three weeks being common in the BDM trials meaning significant reductions in numbers before the assessment of changes to susceptibility can begin. This problem became even more apparent during the anacardic acid and sodium butyrate trials where survival rates dropped in some groups to as low as 16%. This is acceptable depending on the numbers needed and the metric used. Although the use of two assessment criteria are recommended, overall resistance and cercarial count, if complete or significant resistance is expected to be granted lower survival levels are acceptable but when assessing for

pathways that may only partially impact viability of the parasite greater numbers are required. On average it would be advisable to calculate the minimum number of subjects you would need to get a significant result at week 4 and then infect at least double that number assuming an average 50% survival rate over the first 4 weeks.

Whereas the BDM trials were inconclusive at best in regards to whether preventing nuclear myosin driven gene movement could prevent infection it did allow to develop the assay necessary for testing if epigenetic therapies could afford resistance. Alterations in epigenetic factors has been shown to be important for disease development as shown with curcumin induced protection from diabetic nephropathy (Tikoo, *et al*, 2008). Initially focusing on the remodelin and then anacardic acid for the HAT inhibitors the initial remodelin trial demonstrated significant increase in susceptibility with almost 95% of all treated snails shedding a week earlier than normal, the second trial did not show earlier shedding but consistently higher shed numbers with significantly more shed in the fifth week. Unfortunately this was not replicated in the third trial for remodelin but there were survival rate issues with that third trial showing significantly higher death rates prior to shedding than the previous two studies. However, the anacardic acid, another HAT inhibitor does support the same hypothesis that manipulating histone acetylation, specifically preventing acetylation, does result in decreased resistance. The first anacardic acid trial demonstrated significantly greater shed numbers in the fourth week of the trial and consistently shed more over the weeks while the second anacardic acid trial did not shed more but did shed a week earlier. Finally the HDAC inhibitor, sodium butyrate, showed a 100% resistance to infection in two trials indicating that preventing deacetylation results in an increased resistance to infection. This was congruent with other studies using sodium butyrate (SB) that show that it can ameliorate the effects of *C. pseudotuberculosis* infection in rats (Zhou, *et al*, 2019) and decrease salmonella infection in pigs (Casanova-Higes, Andrés-Barranco, and Mainar-Jaime, 2018). SB has also been shown to be potentially effective in treating Epstein-Barr virus (EBV) induced cancers (Westphal, *et al*, 2000) and EBV is currently the only other infection shown to induce chromatin reorganisation (Li, *et al* , 2009). Although in the case of EBV, SB was investigated as a adjuvant to EBV induced cancer therapies not as a direct preventative measure as is the case in this study.

Taken in conjunction the results for the HAT and HDAC inhibitors indicate that preventing acetylation of the histones reduces the resistance of the snail likely allowing progression of the parasite development to happen quicker resulting in either earlier shedding or greater

average number of shedding parasites earlier on. Conversely preventing the deacetylation of histones increases resistance to infection with SB exhibiting a 100% resistance to infection. However, it should be noted that both experiments involving sodium butyrate and anacardic acid had issues with general survivability. This was likely due to changes to the environment the snails were kept in however as remodelin had a similar issue in the last trial where it did not have previously and the latter two experiments were conducted at a newer facility. The snails had previously survived 4 days of exposure to both sodium butyrate and anacardic acid so acute toxicity is unlikely to be an issue but, a long term toxic effect has yet to be ruled out.

It has been demonstrated within this chapter that manipulation of the epigenome does affect *B. glabrata* resistance to *S. mansoni* infection. Considering that inhibiting the acetylation of histones increases susceptibility slightly while preventing deacetylation gives apparent resistance to infection. It could indicate that HAT inhibitors are inhibiting the activation or upregulation of genes involved in protecting the host from infection. Whereas the HDAC inhibitors would be preventing the suppression or down regulation of genes that protect from parasitic infection which would indicate that suppression of the host genome is more advantageous to *S. mansoni* during initial infection. Although certain gene targets are definitely upregulated during initial infection such as *hsp70*, since the diverse role of such stress proteins include chaperoning other partially folded proteins it could be inadvertently affecting the proteome. This could be either by slowing the correct folding of proteins needed to combat the infection or simply the massive upregulation of the *hsp70* gene taking up resources that could be used by more critical immune gene and suppressing the proteome in a similar manner to the genome is being suppressed.

Chapter 4: The effects of ageing on the epigenetic and genome organisation of *Biomphalaria glabrata* and developing *B. glabrata* as a model for investigating the effects of ageing on genome organisation

4.1 Introduction

Ageing is a multifaceted degeneration of an organism over time and includes dozens of co-contributing factors. Due to this the ageing field benefits from having several different models to draw upon when investigating any one facet of the ageing process. *D. melanogaster* for instance with its comparatively short lifespan and multiple tools allowing for gene editing is an excellent organism for exploring the effects of knockdown and knockouts of certain genes on ageing such as *Indy* (I'm not dead yet) (Rogina and Helfand, 2013; Rogina *et al.*, 2000) and *mth* (methuselah) (Petrosyan *et al.*, 2014; Araújo *et al.*, 2013; Lin, Seroude and Benzer, 1998). Whereas murine models have proven invaluable for assessing changes to epigenetics and chromatin (Dou *et al.*, 2017; Cole *et al.*, 2017; Wang, T. *et al.*, 2017). As well as exploring the effects of clearance of senescent cells may have on health as an organism ages (Baker *et al.*, 2016; Chang *et al.*, 2016).

The effects senescence has on nuclear organisation during interphase have been explored in human fibroblasts *in vitro* (Mehta *et al.*, 2010; Bridger *et al.*, 2000) however, replicating these changes seen *in vitro* in an *in vivo* model has proven difficult. What would be required is a model organism that can be easily maintained, be kept in large enough numbers and are similar enough in nuclear organisation to human nuclei with known inducible genes. To this end *Biomphalaria glabrata* a freshwater snail was put forward as a potential organism. Being an invertebrate it meets the criteria for 'replacement' with in the NC3R's framework as a partial replacement, they are comparatively easy to maintain and can be bred in sufficiently large numbers for any experimentation required with comparative ease. Once maintenance is achieved it would be a simple matter to maintain sufficient numbers for each experimental age required and the interphase organisation of *B. glabrata* is more similar to human than other invertebrates (Arican-Goktas *et al.*, 2014) while also possessing genes that are easily inducible such as *hsp70* (Arican-Goktas, 2013) allowing for investigation of cessation of gene movement which is a hallmark of senescent cells *in vitro* (Bridger unpublished data) in an *in vivo* experiment.

One of the targeted genes during *S. mansoni* infection of *B. glabrata* is *actin* (Arican-Goktas *et al.*, 2014). Actin proteins serve several major functions including making up part of the cytoskeleton that supports the cell (Stricker, Falzone and Gardel, 2010). They are also partially responsible for intracellular movement when they interact with another protein,

myosin (Le Goff, Amblard and Furst, 2001; Tsukada, Azuma and Phillips, 1994). Generally filamentous actin acts as a tether or guide line and the motor protein myosin will move along it. In this manner this manner the two protein work in tandem to move organelles or constrict tubules (Ripoll *et al.*, 2018; Buss, Spudich and Kendrick-Jones, 2004). Both actin and myosin proteins have been localised to the nucleus and considering *actin* is targeted for upregulation by the *S. mansoni* parasite and gene relocation is known to occur during infection could these two be linked and if so could myosins also be affected. Nuclear myosin 1β (NM1β), has also been demonstrated to be affected during changes in cell fates from proliferating to quiescent (Mehta *et al.*, 2010), distribution is affected in progeroid syndromes like Hutchinson-Gilford Progeria Syndrome (HGPS) (Mehta *et al.*, 2011) and restoration of myosin can prevent premature senescence of osteoblast stem cells in rats (Zhang *et al.*, 2011). NM1β is also been indicated in roles involving transcription (Philimonenko *et al.*, 2004) and myosin inhibitors like 2,3-butanedionemonoxime prevents induced gene movement (Arican-Goktas, 2013; Mehta *et al.*, 2010). It is therefore possible that changes in chromobility seen in senescent cells may also be a result of alterations to NM1β distribution only these changes, unlike in quiescence, may be irreversible. As such the effects of old age (12 months) were investigated in the snail and how age impacts on chromatin organisation via epigenetics and chromobility via inducible gene relocation and NM1β distribution and how these are comparable to changes seen *in vitro* in senescent human cells (Bridger unpublished data).

4.1.1 Aims

- To assess age related alterations to histone methylation modification distribution
- To investigte if the *hsp70* gene loci is non-randomly relocated in old age for *B. glabrata in vivo*.
- To investigate if after age related relocation of the *hsp70* gene loci that gene loci can be induced to be move via heat shock or infection.
- To assess changes to nuclear myosin 1 beta (NM1β) distribution within nuclei as a result of age.
- To assess whether there is an increase in cellular dysfunction via FISH by prevalence of polyploidy of the *hsp70* loci.
- Comparison of *in vivo* aged *B. glabrata* nuclei to previous data on human senescent cells *in vitro* to establish is *B. glabrata* is a viable model for studying the effects of ageing on genome organisation.

4.2 Methods

4.2.1 Snail Husbandry

See protocols outlines in 2.2.1.

4.2.2 Cell Suspensions for IF

See protocols as outlined in 2.2.2 for how cell suspensions were created for aged snails. In the case of aged snails however due to the greater size of the snail there is inherently more tissue available when sampling so only a single snail was used for each cell suspension. Aged snails were also exposed to both heat shock and parasite infections following the protocols outlined in 2.2.3 and 2.2.4 respectively. However, due to the greater size of the aged snails 6-well plates were used for the individual infections using 3mL of lepple aquarium water and 10 miracida per well for each snail was maintained.

4.2.3 Indirect Immunofluorescence

See protocols outlined 2.2.5 for preparing the slides for IF. Assessment for NM1β staining was conducted on three snails from each group and pattern counting was performed until a single pattern reached 200 and then percentages derived from these data were used.

4.2.4 2D Fluorescence *in situ* Hybridisation

See protocols 3.2.1 for creation of cell suspension for FISH and protocol 3.2.2 for the FISH protocol used and 3.2.3 for how the images were analysed.

4.2.5 Aged related resistance to Infection

Following the protocol outlined in 4.2.2 for infection and 3.2.5 for assessing overall resistance to infection 10, 12 month old (aged), snails were infected with miracidia and split into 2 groups. The control group consisting of 5 12 month old snails and a test group of 5 snails that were treated with 1μM Remodelin for 24h prior to being washed and exposed to the miracidia.

4.2.6 Age related polyploidy

Images from FISH on nuclei derived from the ovotestes of juvenile and aged snails were pooled from three biological replicates each and haploid and negative nuclei were removed. The number of *hsp70* gene loci were then counted in each nucleus and tallied. Each biological replicate contributed at least 90 images to the pool and the pool was of at least 300 images after haploids had been removed. Each replicate was counted separately to allow for a statistical T test to be performed on the overall percentage that each of the three categories made of the overall picture. The three categories used were diploid (2 signals), tri-/tetraploid (3-4 signals) and polyploidy (5≤).

4.3 Results

The field of ageing is an ever growing body of research that encompasses all aspects of biology. Investigating the effects *in vivo* on genome organisation however, is hampered by the current models either due to a combination of ethical and logistical issues with mammalian models such as mice or the lack of similarity in genome organisation or ageing process in invertebrate models such as fruit flies. *B. glabrata*, although an invertebrate model, does not undergo a metamorphic stage in its development and consequently are not made up of mainly post-mitotic cells as with the commonly used invertebrate ageing models such as *D. melanogaster*. Similar benefits arise in comparison to mammalian models as *B. glabrata* has a comparatively shorter lifespan, is easier to care for and being aquatic it is easier to test compounds for their effect on genome organisation. Taking into consideration the previous factors this study took the comparatively similar genome organisation of *B. glabrata* to human organisation and investigated whether similarities to that seen in human cells grown *in vitro* to senescence could be drawn in the aged organism.

4.3.1 Age Related Epigenetic Changes

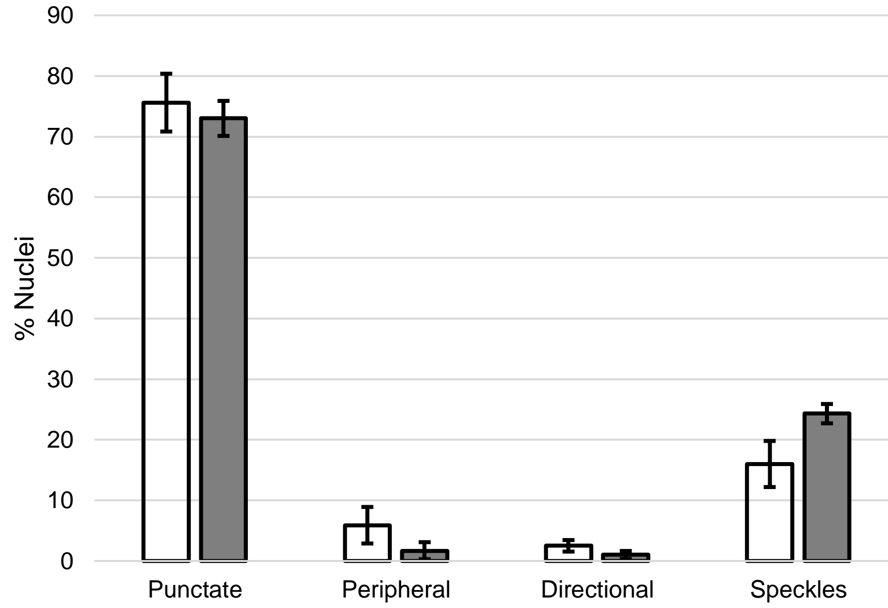
Genome organisation is a complex interplay of several factors one of these factors is changes to the histone proteins that make up the nucleosome and subsequently affects how tightly compacted the chromatin is, how accessible genes are to transcriptional protein complexes and potentially chromatin organisation. Here three such markers are investigated, H3K27me^3, H3K79me^3 and H4K20me^3. Five patterns [Fig 2.8] were initially identified in juvenile snails these being,

1. Punctate, patterning exhibiting multiple small foci distributed throughout the nuclei in an even spread.
2. Speckles patterning which showed larger distinctive foci distributed throughout the nuclei that were unevenly distributed.
3. Peripheral, pattern staining that exhibited a continuous stain around the periphery of the nuclei with little to no staining in the centre of the nuclei.
4. Peripheral Foci, pattern staining where distinctive large foci like those in the speckles patterning are located primarily or exclusively at the peripheral of the nuclei.
5. Directional, pattern staining exhibiting heavy staining in one area of the nuclei encompassing part of the peripheral and some of the internal volume,

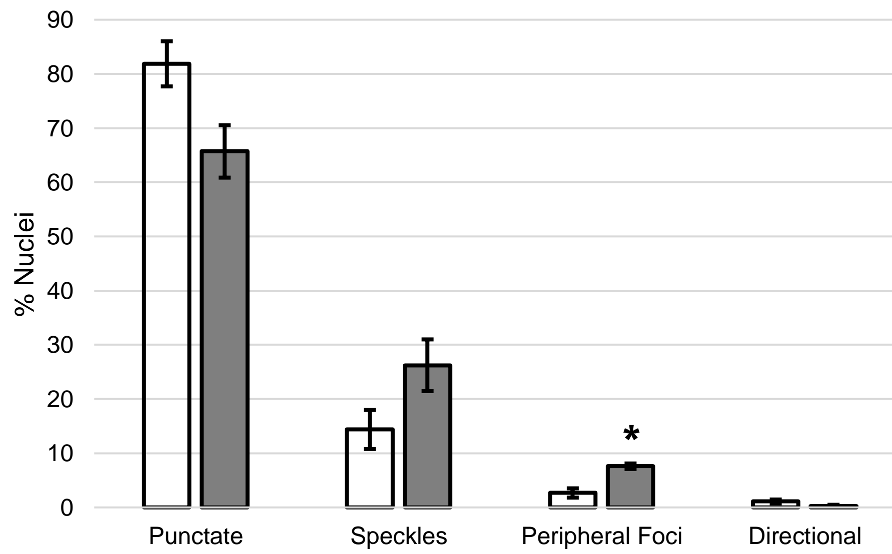
These are represented in Fig 2.1.

The same patterns were discerned in the aged snail and the aged snails response to heat shock were investigated, the results demonstrated in Fig 4.1, and compared to the response seen previously in juvenile and adult snail controls and after heat shock for H3K27me^3 [Fig 4.2], H3K79me^3 [Fig 4.3] and H4K20me^3 [Fig 4.4]. It is a well-established fact that as organisms age they become frailer and less able to adapt to changes that younger organism can tolerate with comparative ease, such changes include changes to histone (Nelson *et al.*, 2016), lamins (Dou *et al.*, 2017) and DNA methylation (Meer *et al.*, 2018; Welberg, 2014). With this comparison some conclusions could be drawn as to whether part of what leads to this fragility is a change in epigenetic response in cells of older organisms limiting the kind of response to stress that can be elicited.

A



B



C

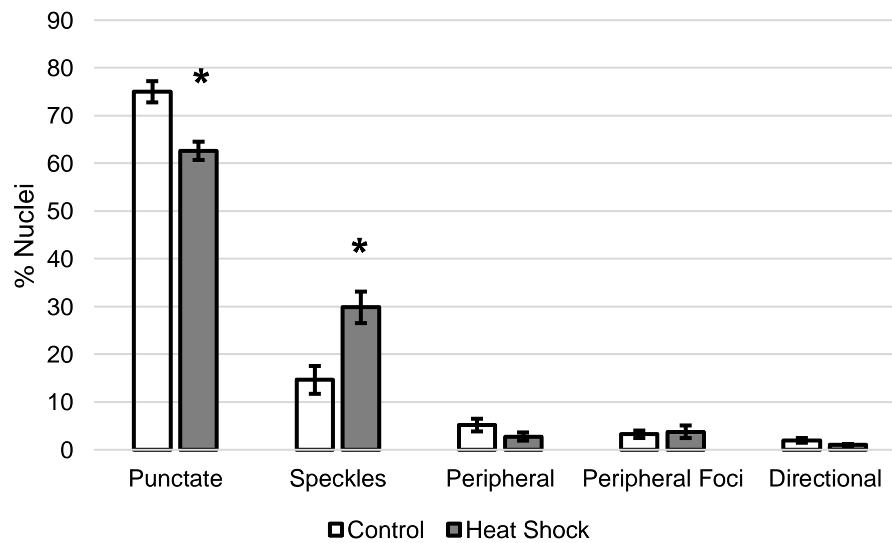


Fig 4.1 The charts show the average percentage of nuclei exhibiting the patterns outlined in Fig 4.1 present in each of the histone modifications tested A) H3K27m^3, B) H3K79me^3 and C) H4K20me^3 for both Control and Heat Shocked Aged snails. * denote patterns that exhibit statistically significant change in distribution p-value <0.05, error bars = S.E.M. n= 1000 nuclei, in triplicate.

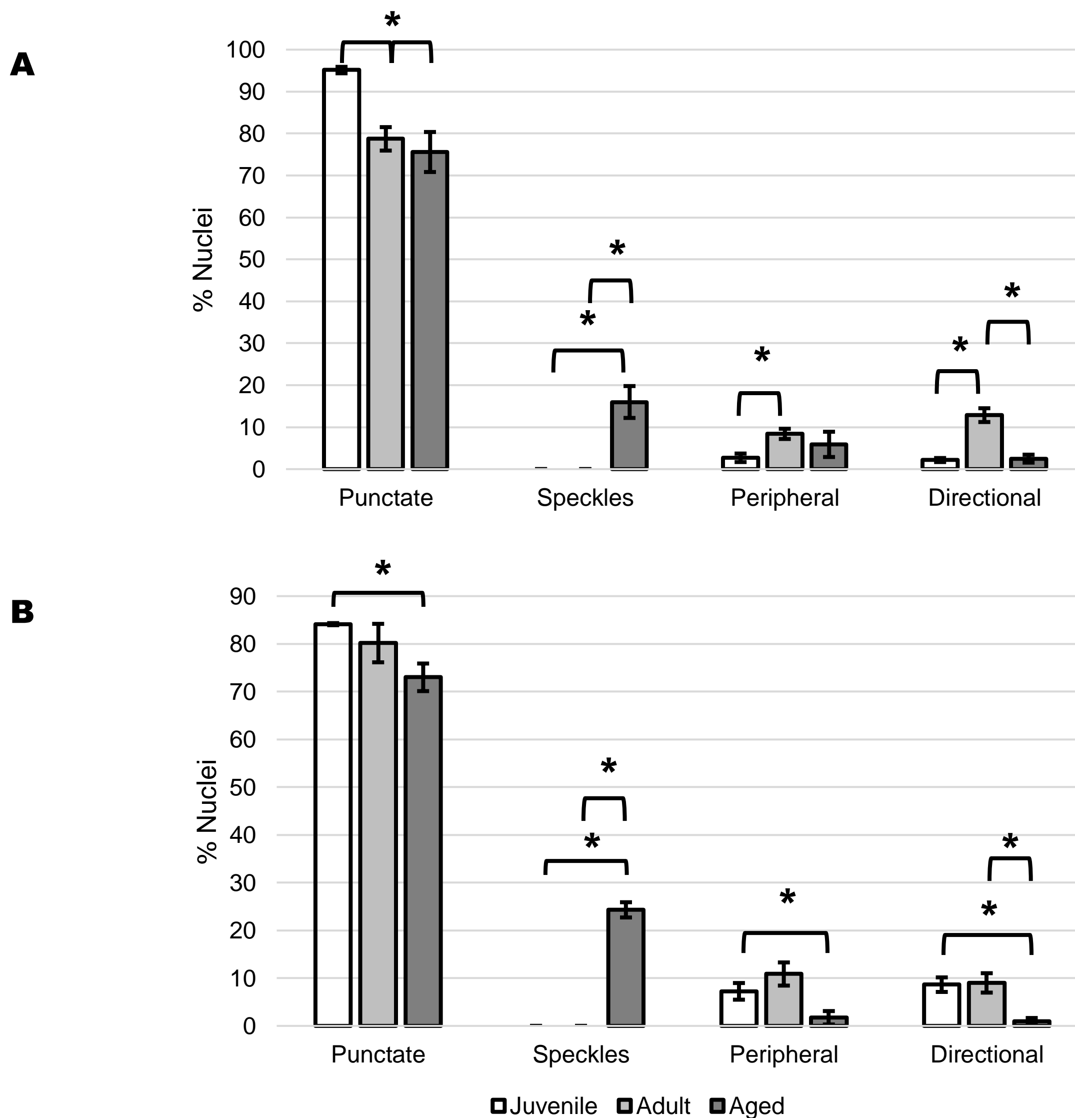


Fig 4.2 Representation of pattern population distribution for H3K27me^3 modifications across three age groups, juvenile (4 weeks), Adult (3 months) and Aged (12 months) in one of two conditions A) Control and B) Heat Shocked. * denote patterns that exhibit statistically significant change in distribution, p-value <0.05, error bars = S.E.M. n= 1000 nuclei, in triplicate.

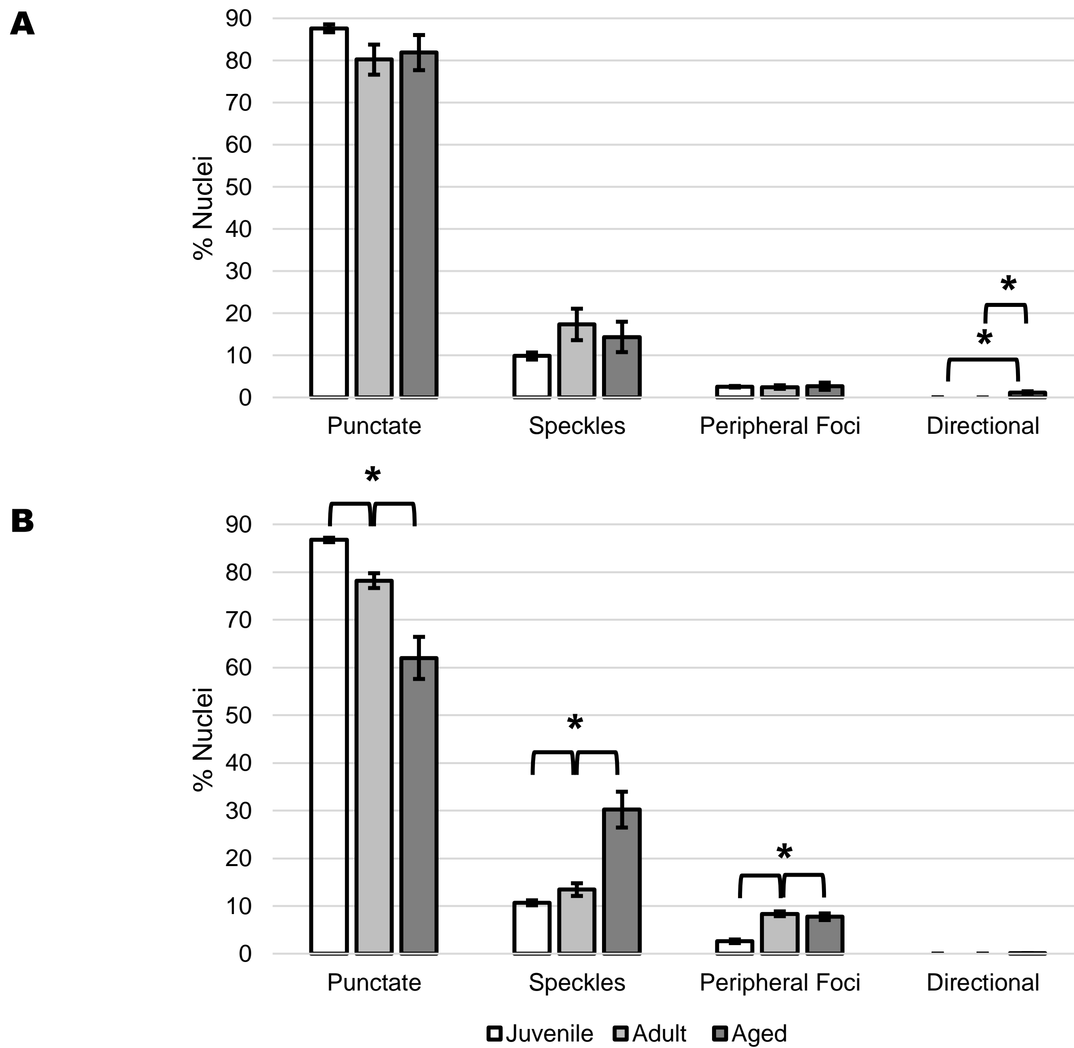


Fig 4.3 Representation of pattern population distribution for H3K79me^{3} modifications across three age groups, juvenile (4 weeks), Adult (3 months) and Aged (12 months) in one of two conditions A) Control and B) Heat Shocked. * denote patterns that exhibit statistically significant change in distribution, p-value <0.05, error bars = ± S.E.M. n= 1000 nuclei, in triplicate.

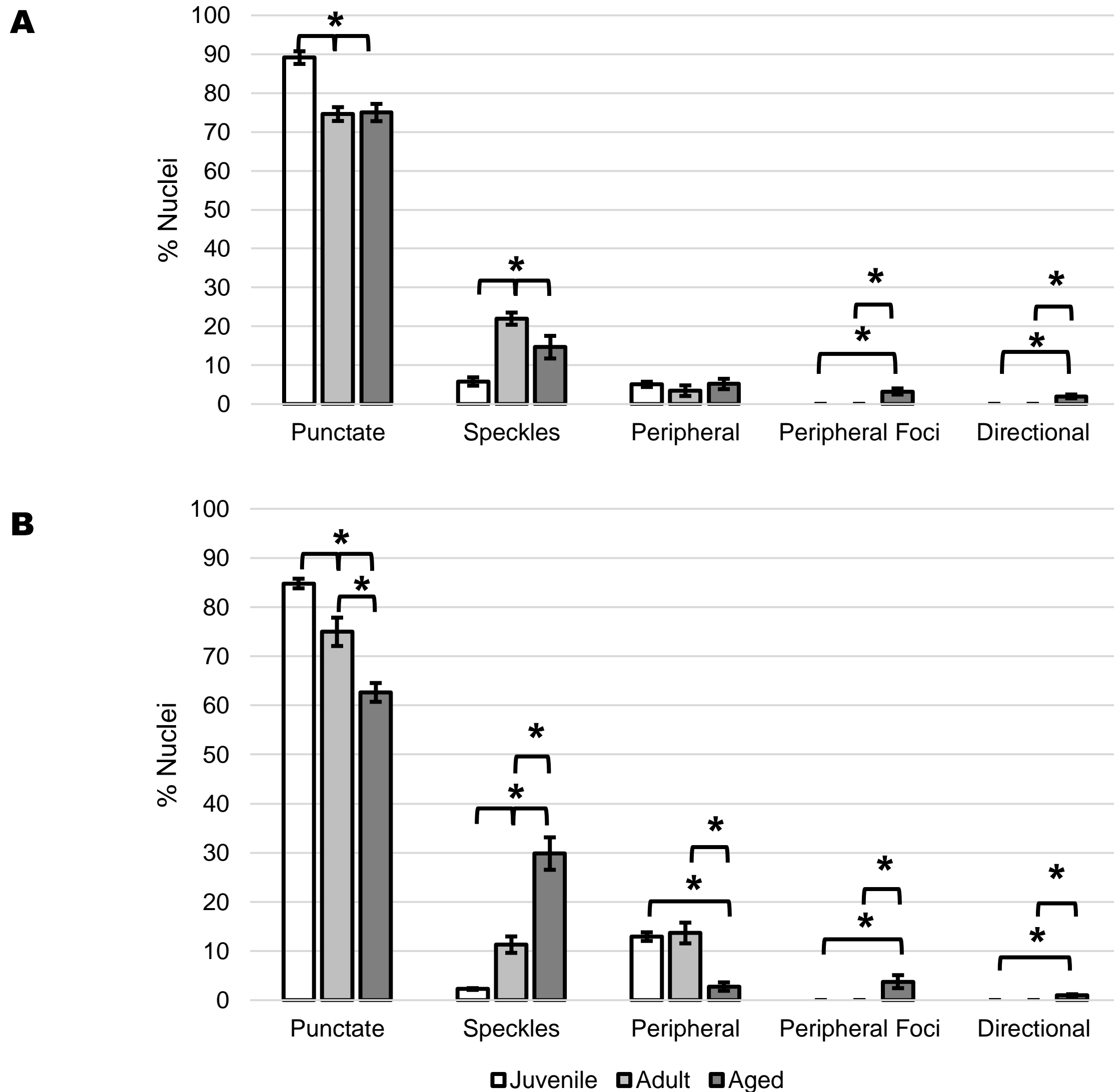


Fig 4.4 Representation of pattern population distribution for H4K20me³ modifications across three age groups, juvenile (4 weeks), Adult (3 months) and Aged (12 months) in one of two conditions A) Control and B) Heat Shocked. * denote patterns that exhibit statistically significant change in distribution when comparing either adult or aged cohorts to juvenile cohort, p-value <0.05, error bars = S.E.M. n= 1000 nuclei, in triplicate

After scoring the patterns of histone modifications the most noticeable alteration due to age in the snails are an increased number of patterns being exhibited that were not seen in the juvenile cohort. H3K27me³ has started to exhibit speckles, H3K79me³ show a directional pattern and H4K20me³ demonstrate both peripheral foci and directional patterns. Yet a key difference between juvenile and aged snails is most obvious in H3K27me³ patterning. The speckled patterning which H3K27me³ juveniles did not exhibit is now quite abundant, making up 16% of all observed patterns in the control. Considering the low abundance of the new

patterning in the $H3K79me^3$ and $H4K20me^3$ cohorts it would seem unlikely that this could be merely a result of damaged or mutated cells. This was further supported when comparing the speckles patterning in the other two modifications with $H3K79me^3$ and $H4k20me^3$ displayed a 4% and 8% increase, respectively. Similarly when comparing all three modifications to one another the percentage of speckles patterning in each is quite similar to one another ranging from 14-16% in the controls and 24-30% in the heat shocked snails. Furthermore, all of the patterns showed the same increase in speckle staining after heat shock, even if previously there had been a decrease in speckle staining as with $H4K20me^3$. When comparing the aged cohort to the juvenile cohort this change in speckled staining proved to be statistically significant in all but the $H3K79me^3$ controls. Similarly reductions in the punctate staining were also significant in all but the $H3K79me^3$ controls indicating that the majority of this change is likely a shift from punctate staining to speckle staining.

4.3.2 Alteration in gene positioning

The *hsp70* gene loci positions for the BB02 wild type susceptible strain of *B. glabrata* has been previously established to be internally positioned and shifts to an intermediate position following either heat shock (Arican-Goktas, 2013) or infection by *Schistosoma mansoni* [Fig 3.10]. Previous work performed in the Bridger lab also indicates that human cells passaged into senescence will undergo significant chromosomal rearrangements upon becoming senescent (Mehta, I. S. *et al.*, 2007; Bridger, J. M., Boyle, Kill and Bickmore, 2000) and once this occurs no further rearrangement of genes or chromosomes are possible. To establish *B. glabrata* as a model for *in vivo* genome organisation changes associated with ageing the ground work required at least one known gene which could be reliably induced to move. In this respect there is the *hsp70* loci that has been induced to move via heat shock (Arican-Goktas, 2013) and infection by *S. mansoni* [Chapter 3]. Knowing the canonical position for the BB02 susceptible strain to be internally positioned it needed to be established what the position was for the aged cohort Figure 4.5 demonstrates where the canonical *hsp70* gene loci position is in the aged snail cohort as well as the position of the gene loci following either heat shock or *S. mansoni* infection, these were compared to the previously established positions.

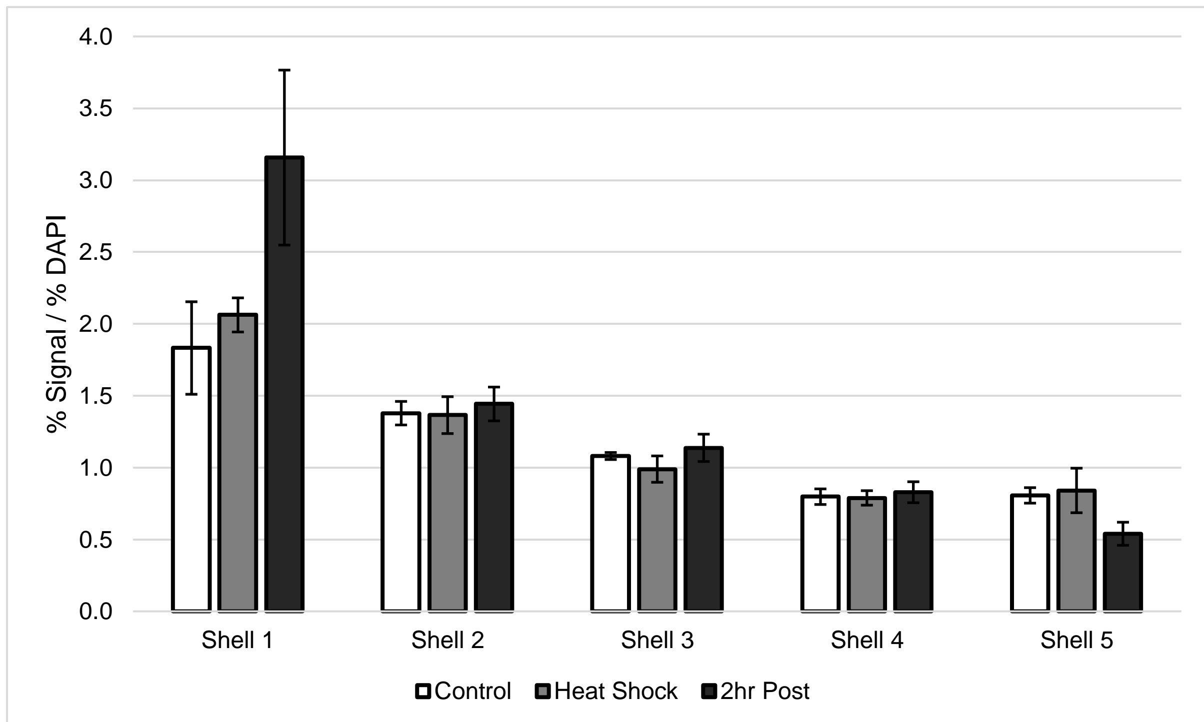


Fig 4.5 The comparative gene position of the *hsp70* loci in aged (12 month old) snails, comparing control to heat shock and *S. mansoni* infection both known induces of *hsp70* gene loci movement. There is no statistically significant change in comparative gene position post heat shock or *S. mansoni* infection. n ≥ 50 nuclei, in triplicate.

As can be seen in Fig 4.3 the *hsp70* gene loci for the aged cohort is located at the periphery of nuclei showing a marked difference from the canonical position of the juvenile BB02 strain which is located at an internal position. Similarly unlike with the juvenile cohort where both heat shock and *S. mansoni* infection are capable of inducing gene movement we can see there is no statistically significant change in gene positioning, using a two-tailed student T-test assuming equal variances, between the three groups indicating that either the gene cannot be moved or does not need to be moved. However, taking into account what is known about human senescent cells in culture it would not be unreasonable to suggest that after this observeable gene rearrangement that it is not possible to move the genes. This replicates what is seen *in vitro* in human fibroblasts in an *in vivo* model.

4.3.3 Nuclear Myosin 1β

Nuclear myosin 1β (NM1β) is a target of 2,3-butanedione monoxime (BDM) previously used to prevent gene movement *in vitro* in the Bridger lab, although as a reversible drug the effects are not long lasting enough to prevent infection as borne out during the BDM drug trial (Chaper 3). However, previous work has shown that in human cells passaged into senescence that NM1β is affected in ageing cells. Combine this with the changes seen in epigenetic response which could potentially be linked to chromatin mobility the distribution of NM1β was investigated in both the juvenile and aged snails. Four patterns of NM1β distribution were identified as shown in Fig 4.6, however though similar patterns were seen in both the juvenile and aged cohorts the intensity of staining was significantly different with the foci seen in both peripheral foci and speckles patterning being lower in number in the aged cohort and the punctate patterning in the aged cohort appearing to be almost negative in the majority of cases.

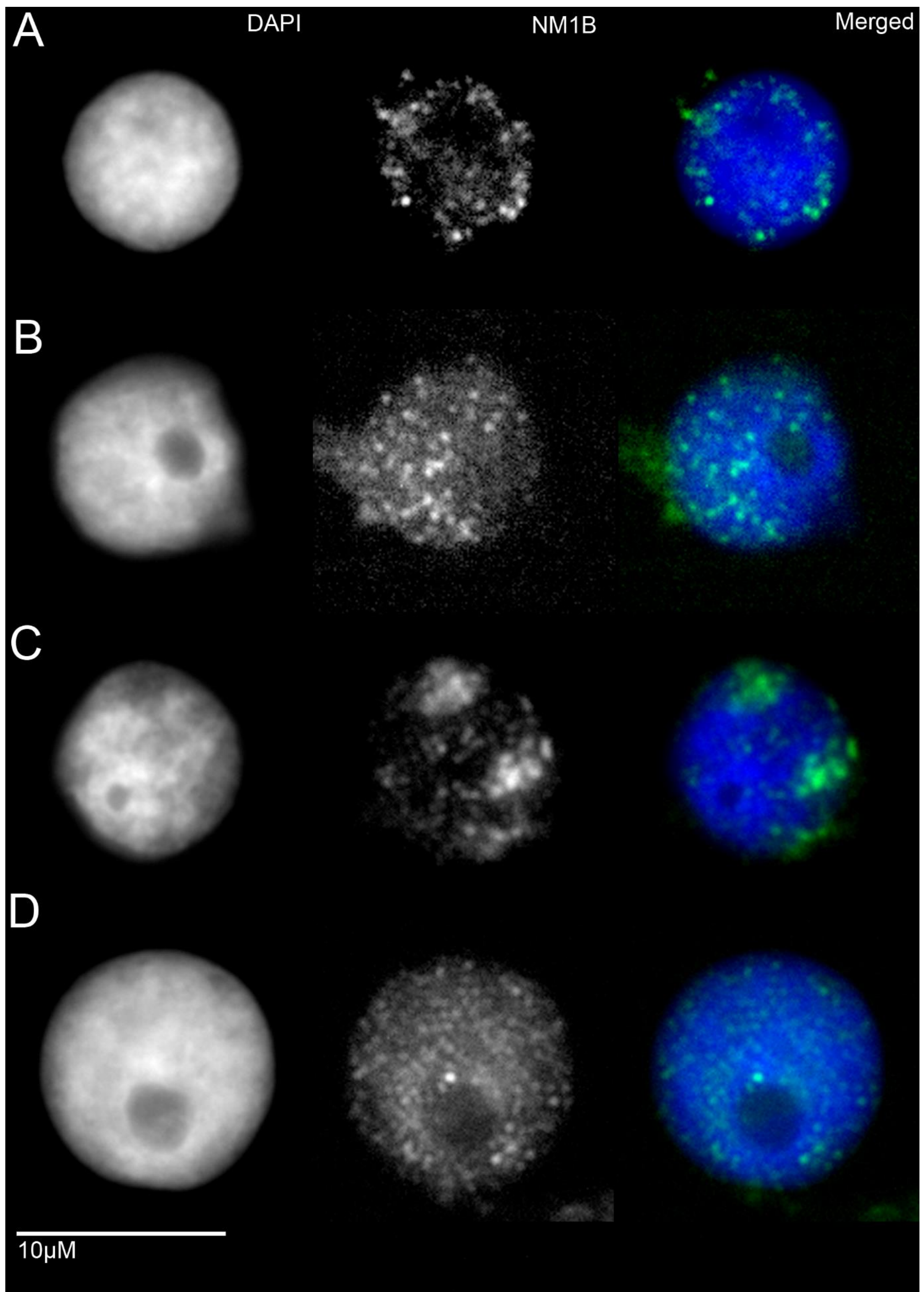


Fig 4.6 Representative images of patterns seen with NM1β antibody staining. A) Peripheral Foci, larger distinct foci that are positioned towards the periphery of nuclei B) Speckles, large

distinct foci distributed throughout nuclei C) Large Regions, concentrated asymetrical staining of different areas of nuclei D) Punctate, fine small foci evenly distributed throughout the nuclei

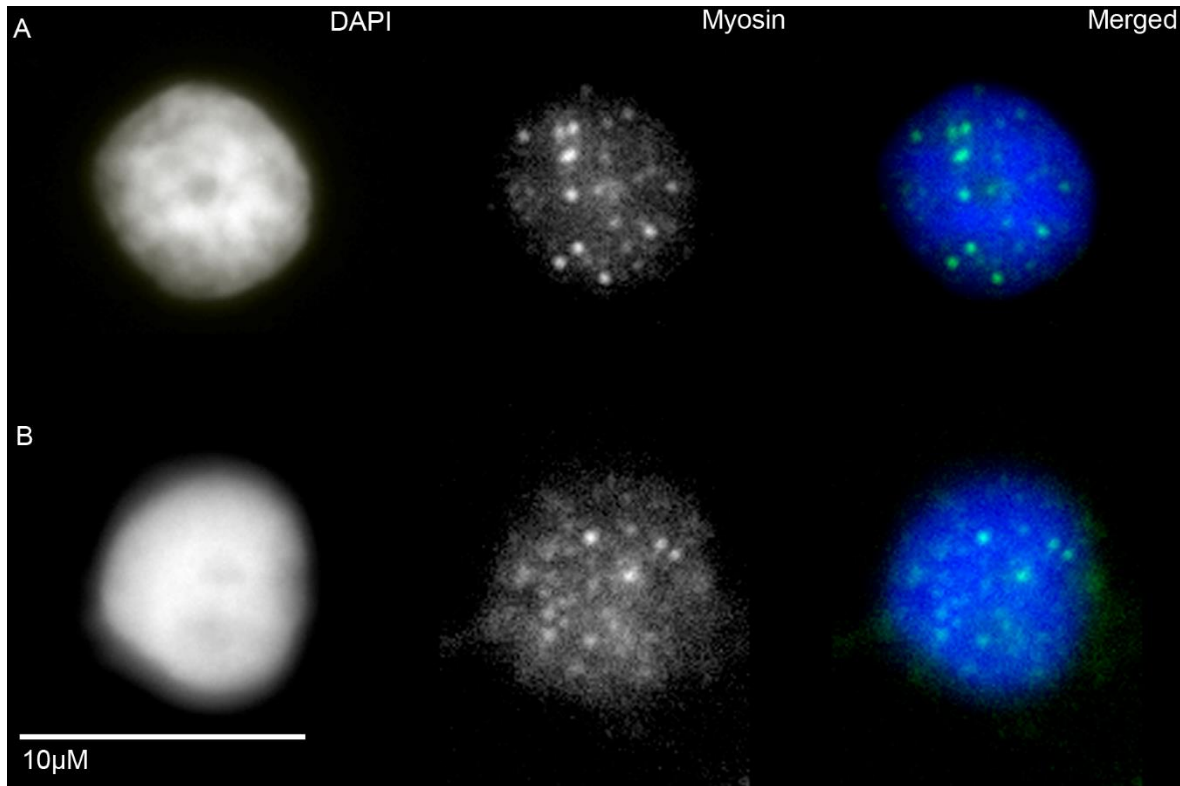


Fig 4.7 Representative images exhibiting the difference between speckles in the juvenile and aged cohorts taken at 0.1 shutter speed and 1 gain for DAPI and 0.5 shutter speed 5 gain for the mysoin image. A) Juvenile snail nuclei speckle patten showing multiple large distinct foci. B) Aged snail nuclei speckle pattern showing fewer foci that appear duller in comparison to juvenile snail nuclei

The overall pattern distribtuion does seem to change significantly when comparing the juvenile to the aged snail as shown in Fig 4.8. This alteration could be indicative of a decline in NM1β present in the senescent cells which, if this is indeed part of a nuclear motor meant to facilitate chromatin mobility this could explain the apparent absence of such mobility in the aged cohort. Further study is certainly warranted in this investigation especially since previous work shows NM1β disruption in *in vitro* scenescent cells.

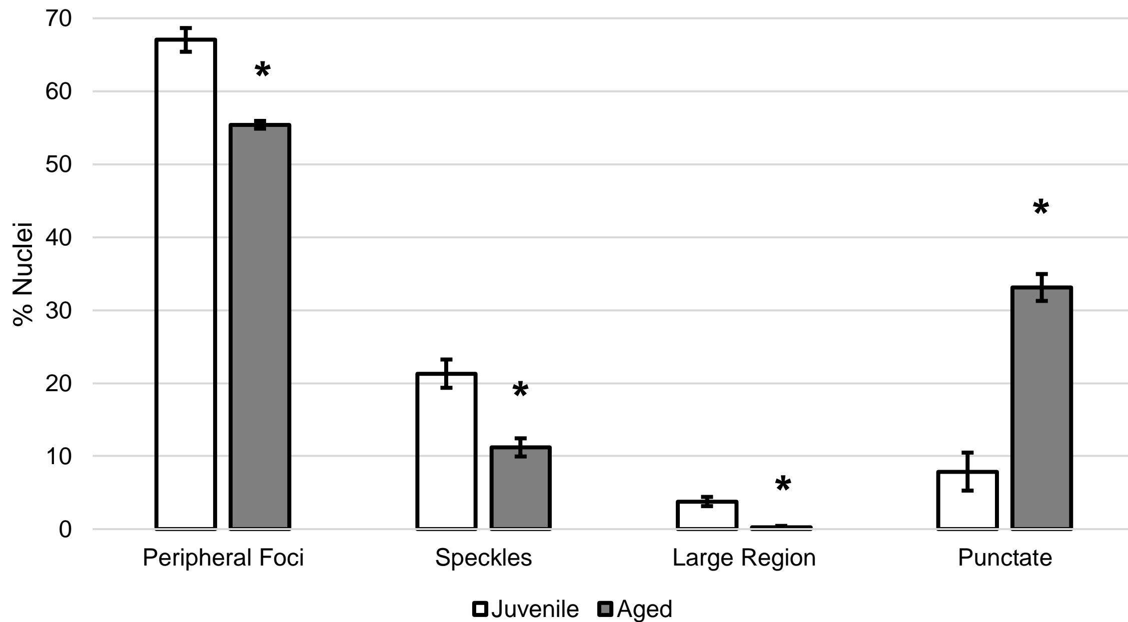


Fig 4.8 Comparison of NM1β pattern distribution between juvenile (n^1= 309, n^2= 285 and n^3= 302 nuclei) and aged (n^1= 367, n^2= 361 and n^3= 355 nuclei) snail cohorts. * denote patterns that exhibit statistically significant change in distribution p-value <0.05, error bars = S.E.M.

4.3.4 Infecting Aged Snails

Snail susceptibility to infection has been shown to be affected by the age of the snail with young adult snails exhibiting greater resistance to infection this however reverts with old age (Richards and Minchella, 1987; Richards 1977). It has also been demonstrated that snail size can affect snail suceptibilty to infection, with larger snails being less suceptible (Niemann and Lewis, 1990). The previous work by Richard C. S. and Minchella (1987) observed snails up to 9 months which had become suceptible once more in a strain with variable adult suceptibility. It was ascertained in the BB02 strain that gene positioning in 9 month old snails differs from that of the aged (12 month) cohorts, this is shown in figure 4.9 which demonstrates an intermediate gene position in control snails, this differs from both juvenile, which is internal, and aged, which is peripheral. Consequently this work was the first time that the suceptibility of such old snails had been investigated and was using the BB02 snail strain to compare survival and resistance between juvenile and aged snails. Normally infections are done with juvenile BB02 snails to ensure an infection rate of ≥95% this drops as the snails become young adults. Now with the aged snails there are three possible outcomes, further increased resistance with age, same susceptibility as seen in adult snails or increased susceptibility to infection reverting to back to juvenile suceptibility. The main factor of consideration here is the advanced age of the snail although true that

young adult snails have an increased resistance over juvenile snails the exact mechanisms are not well understood in susceptibly strains. It could be a function of sheer size which the aged snail certainly has over normal adults. However, with age comes a reduction in function in many key areas including immunoresponce which could mean that the age snail exhibits an increase in suceptible in comparison to younger adult snails. Conversely it has also shown that gene movement is not induced in the aged snail so dependent on how critical this is to infection this could also affect the chances of infection. Fig 4.10 shows the comparative survival and resistance rates of the two groups of snails while Fig 4.11 shows the average cercarial shed from aged snails.

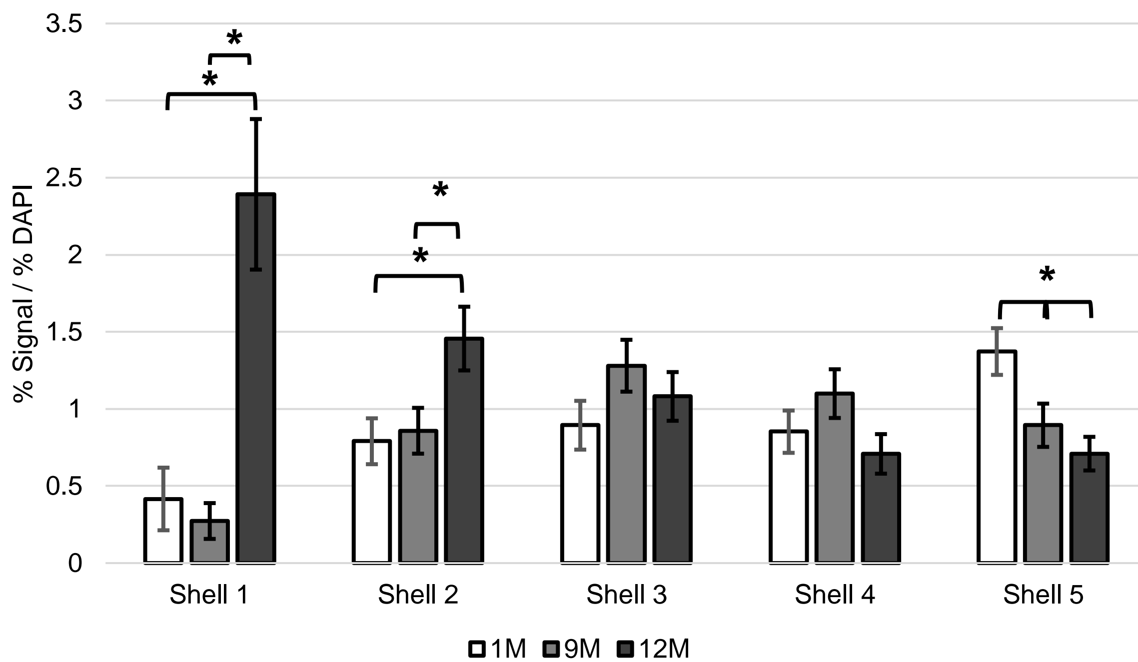


Fig 4.9 Relative gene position of the *hsp70* loci in BB02 strain *Biomphalaria glabrata* of differing ages, 1 month, 9 month and 12 month. * indicate the shells that show statistically significant with a p-value < 0.05. error bars = S.E.M. n ≥ 50 nuclei images for each age group.

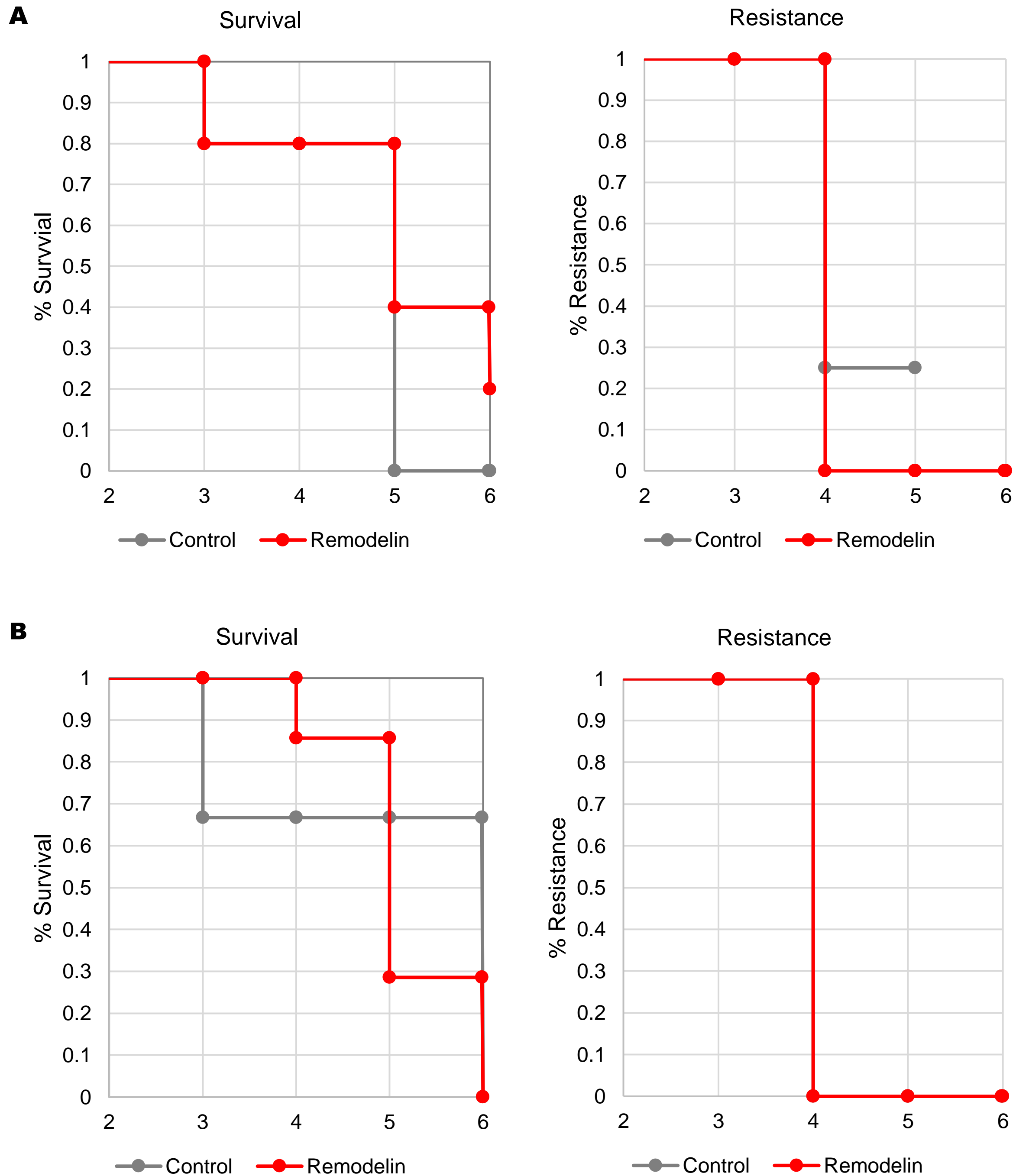


Fig 4.10 Comparison of control and remodelin treated snails' survival and resistance in two age groups over six weeks. A) Aged snails' ($n^{control}$= 5 snails, $n^{Remodlein}$= 5) survival and resistance B) Juvenile snails' ($n^{control}$= 6 snails, $n^{Remodlein}$= 7) survival and resistance.

The aged snails did not survive long following infection by *S. mansoni* in either group. Similarly there seemed to be no change in resistance to infection compared to juveniles. The single non-shedding snail present in the control group at week 4 had died by week 5 so

it is uncertain whether it had resisted infection or simply was too weak to allow the maintenance of an infection to the point where viable cercariae were shed. The treatment with remodelin was initially to see if it could reduce the susceptibility of the aged snails as has been shown with HAT inhibitors in the event that the aged snails still exhibited the increase resistance shown in adult snails. It is unclear whether this ultimately affected the snails although the remodelin treated snails did survive infection better, with 40% surviving until the 5th week when all of the controls had perished there is also the fact that 100% of the snails exhibited active infection at the 4th week. Remodelin also potentially affected the development of the *S. mansoni* infection as the treated snails seemed to shed significantly more cercariae than the control group [Fig 4.11]. When comparing the juvenile survival and resistance rates to those of the aged snails there was also no significant difference discernible between the two groups, either with or without treatment.

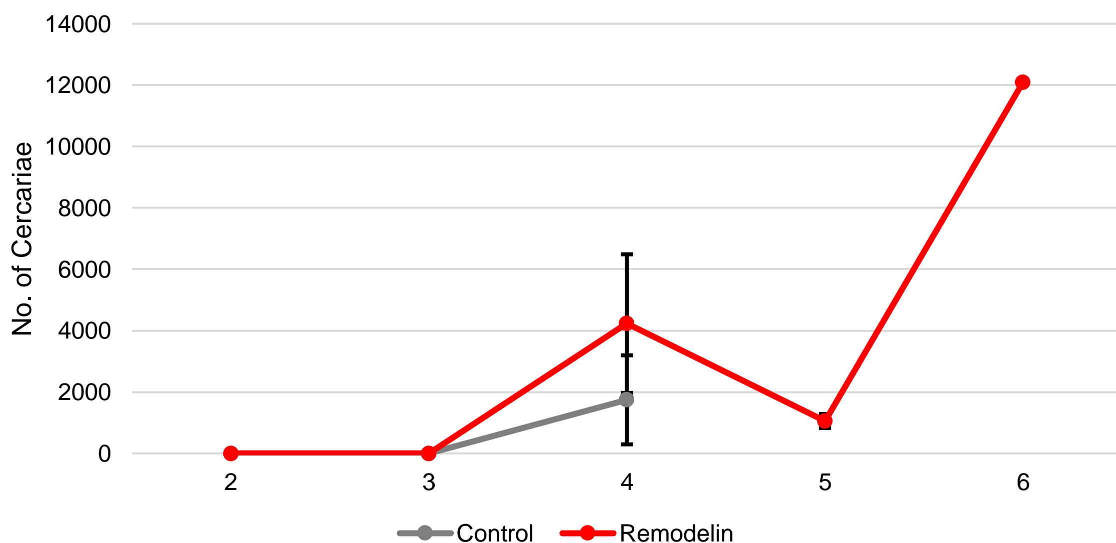


Fig 4.11 The average cercariae shed from each group during a 1 hour window once a week

The average cercarial shed numbers as shown in Fig 4.11 shows quite a dramatic two-fold difference in the average number of parasites shed on the 4th week. However, due to a wide variance in numbers of both groups this caused an overlap that could not be seen as statistically significant. However, it can be concluded that although mature snails are harder to infect this gain in resistance is something that diminishes as the snail grows older meaning that the parasite would have two periods where it could potentially infect the snail easily as indicated in previous work (Richards and Minchella, 1987; Richards 1977) and potential loss of chromobility at extreme age investigated does not impact on infection of

aged snails. This could also indicate that *B. glabrata* could be of use in studying the decline of innate immunity with age if it can be demonstrated that it is in fact a decline in their immune system that precipitates their increased susceptibility to infection in the aged cohort. Remodelin in the aged cohort exhibited no increase in cercarial numbers or earlier shedding that it has done in juveniles. Remodelin was originally developed as a medication for alleviating the symptoms of a partial progeroid syndrome Hutchinson-Guilford Progeria Syndrome (HGPS) and although only slightly and not of significance in this trial the aged snails treated with remodelin did have slightly better survival than the control in the aged cohorts.

4.3.5 Polyploidy Increases with Age

Another hallmark of ageing is an increased number of dysfunctional cells, normally these like senescent cells can be cleared by the immune system however, just as with senescent cells as the immune system decline they can start to accumulate in the body. Some of these dysfunctional cells could potentially become benign growths or cancerous. Defining such cells within a model organism would require significant investigation. Here is started a basic investigation into the ovotestes of the snail looking at an obvious marker for cellular dysfunction, the accumulation of aneuploidy. A cell will develop aneuploidy when it is unable to enter mitosis and divide properly, normally such cells would stop replicating their DNA and enter a senescent state however, with mutations this control can be bypassed and cells can continue to erroneously duplicate the DNA growing larger and causing issues for the cells around it as it disrupts the microenvironment, can no longer assist in proliferative repair of the organ and potentially has reduced to no function further compromising the tissue. As the tissue examined in this instance is derived from a cell pool taken from the ovotestis the accumulated aneuploidies could be from several potential processes either linking to cancer or fertility issues with age as cells could be of either somatic or germline origin.

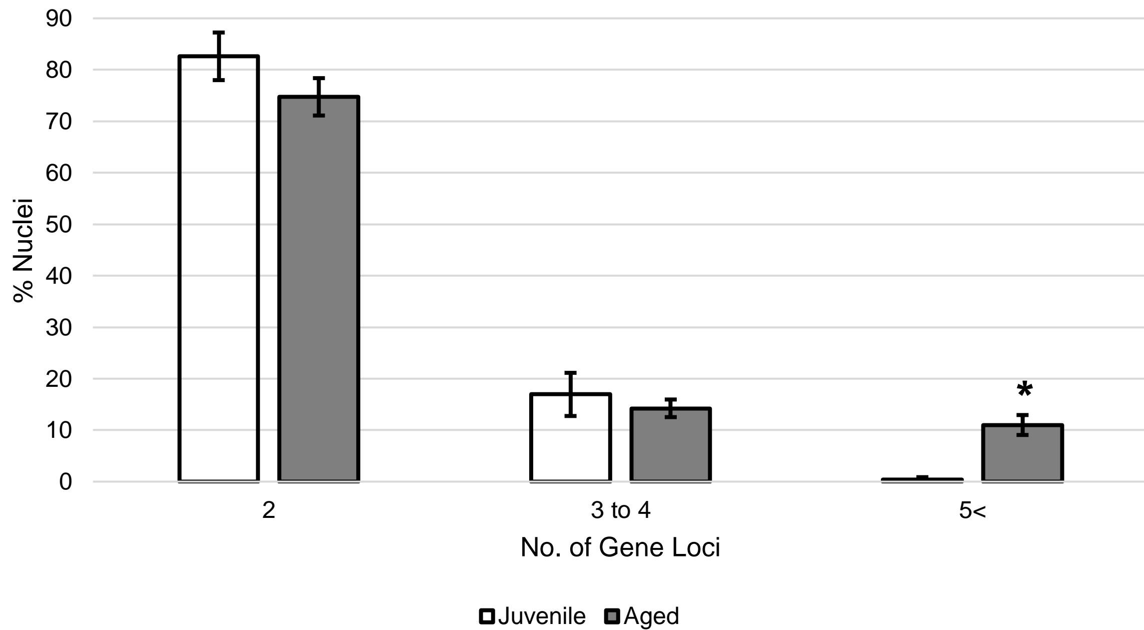


Fig 4.12 This demonstrates the percentage of different ploidy states found in tissues from control juveniles (n^1= 93, n^2= 127 and n^3= 154 nuclei scored) and aged (n^1= 148, n^2= 147 and n^3= 170 nuclei scored) cohorts during FISH experimentation comprising at least 300 images taken and assessed after haploid and null images had been removed from each set pooled from 3 biological replicates each. * denotes statistically significant change in number of a ploidy state between juvenile and aged p-value <0.05, error bars = S.E.M.

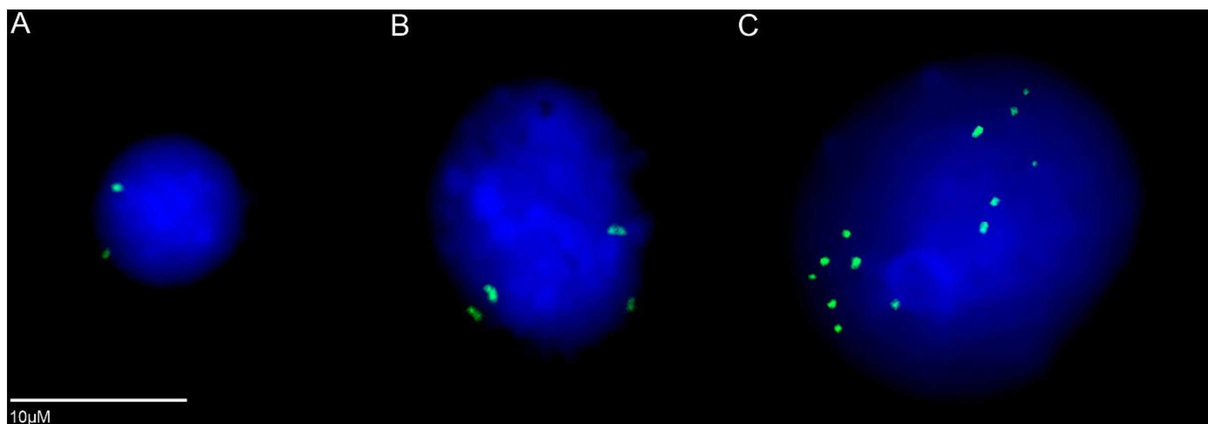


Fig 4.13 Representative FISH images of nuclei exhibiting the *hsp70* loci (green), A) Diploid nuclei showing two loci, B) Tetraploid nuclei with four loci and C) Polyploid nuclei with greater than 5 loci, in this example potentially up to thirteen loci are present in a single nuclei.

As can be seen in Fig 4.12 the vast majority of cells still maintain their diploid state with a smaller percentage exhibiting tri- or tetraploid which are likely preparing for mitosis. It is also possible that some of these numbers could be made up of dysfunctional germline cells that have not split properly but at this point that would be hard to verify however it does clearly demonstrate that cellular dysfunction is limited within the juvenile cohort. In comparison the aged cohort shows no statistically significant difference in either the potentially normal states categorised above. However, when comparing the obviously dysfunctional cells, those with severe polyploidy of 5 or greater copies of the *hsp70* gene, as demonstrated in Fig 4.13C, there is a statistically significant increase in the number of cells that exhibit this characteristic. Now since the ovotestis is the tissue used for these tests this may simply a function of decreasing fertility as a result of malfunctioning germline division rather than tumorigenic in nature but, it does open the door for *B. glabrata* to be used for future investigations for either the potential effects of ageing on fertility, on tumorigenesis or both.

4.4 Discussion

The objective of these experiments was to ascertain if *B. glabrata* was potentially a viable model for the study of genome organisation in the field of ageing. It began with investigating whether global epigenetic changes, via chromatin modifications, which could be indicative of altered genome organisation could be identified between a juvenile and aged cohort. From previous work in the field of ageing in mammalian models it is known that $H3K27me^3$ (Ma *et al.*, 2018; Dou *et al.*, 2017) and $H4K20me^3$ (Nelson, *et* al, 2016) are affected as organisms age and $H3K79me^3$ from previous work in the adult snail was known to be relatively unaffected by stress such as heat shock or cold shock while being affected by infection making it a relatively stable marker for assessment of age related stress. Initial investigation revealed that all three showed some level of aberrant staining with patterns that were not present in the juvenile cohort appearing in the aged cohort. This in itself was not unexpected. As an organism ages systems breakdown and become more error prone which could easily allow for a small number of aberrant patterns to appear in aged tissue. The numbers seen in $H3K79me^3$ and $H4K20me^3$, aside from the appearance of new abberant patterns, initially seemed to follow a similar distribution to that seen in the juvenile cohort. $H3K27me^3$ highlighted the biggest difference in age as it also showed a new pattern. However, this could not be accounted for by aberrant patterning due to a few dysfunctional cells as it made up a significant proportion of the patterns in the aged cohort. Then on closer inspection of the aged cohorts for $H3K79me^3$ and $H4K20me^3$ the same change in patterning

was observable, although both had previously had the speckled pattern it now made up a greater proportion of the population, significantly so in all but one case. Also when comparing the three histone modifications to one another the speckled pattern made up a similar percentage of the aged cohort in both control and heat shocked samples with the same increase seen in speckle patterning after stress. This indicated that there was a greater mechanism at play that was exhibiting decreased functionality that could potentially be affecting genome organisation as a whole.

The increased population of speckled patterning within aged cohort snails for multiple histone modifications could indicate that although histone modifications were occurring in specific nuclear areas the indicated change to chromatin organisation they would normally elicit was being inhibited. This was further supported by the increase in speckled patterning exhibited after heat shock. This inhibition of chromatin reorganisation but not histone modifications is indicative of an inhibitory factor related to active transport of chromatin within nuclei. This meant that potentially the modificantion was being made to the histones but what would then read it or was responsible for causing the movement was either no longer active or losing functionality. This would be likely indicate a disruption of the nuclear motor as as such inhibition of movement has been observed when nuclear myosin is disrupted (Kulashreshtha, et al 2016).

To test this hypothesis FISH and gene location was used to compare the gene signals position of the aged cohort to what had originally been established in juveniles. This indicated that a major change had occurred in genome organisation between the juvenile and aged cohort. As the gene positioning had migrated from an internal position to a peripheral position with significantly more gene signal now being located at or near the nuclear membrane. This is similar to what is seen in senescent human cells *in vitro* as significant genome reorganisation occurs in the transition from proliferative to senescent (Bridger *et al*, 2000). The aged cohorts were exposed to stressors capable of eliciting gene movement in juvenile snails, heat shock and *S. mansoni* infection, however exposure to such stress did not elicit gene movement in the aged cohort. This means that either the gene does not need to be moved or cannot be moved to induce an infection. In this instance it would be slightly more prudent perhaps to say the gene could not be relocated, this is because with the histone modification there has already been demonstrated there are potential inhibitory effects of ageing in regards to chromatin organisation. It has also been

demonstrated that after senescense induced genome reorganisation in human cells *in vitro* that further gene movement is not possible (Bridger unpublished data)

Having confirmed both a change to the epigenetic markers and chromobility the next step was to see if there are other changes associated with senescence *in vitro* that are replicated *in vivo* in the aged *B. glabrata* cohort. Another known altered factor within the senescent cells is NM1β (Mehta, *et al*, 2010) which our lab hypothesise may form part of an actin-myosin nuclear motor within the nucleus, responsible for rapid genome reorganisation. The initial testing for this has shown that there is potentially a difference in pattern distribution, with the more common peripheral foci distribution shifting to a more punctate distribution however, it should be noted here that the punctate distribution observed in the aged cohort exhibited significant reduction in fluorescence appearing dull to the point where nuclei could be mistaken for being negative at first glance. This was also the case with the peripheral foci in the aged cohort where brightness and number of foci are both diminished although not to the extent seen in the majority of the punctate patterned nuclei. This could be an indication of decreased levels of NM1β being present in the cells or being present in a more inactive state. If this is the case it would make sense of what is observed in the histone modifications as the number of nuclei that show the speckle staining after heat shock is in the region of 24-30% and the number of punctate cells that appeared nearly negative is 32%. Considering after a global stress response like that caused by heat shock one would expect all the nuclei with limited functionality to be highlighted as such it is interesting that the percentages here are so similar. Although, not conclusive, it is supportive of the idea that NM1β may play a role in genome reorganisation, especially when added to this is evidence that inhibition of nuclear myosins in general can prevent gene movement from occurring when inhibition is in effect (Mehta *et al*, 2010, Kulashreshtha, et al 2016)

Considering gene movement did not seem to be inducible in the aged cohort it seemed reasonable to see if they could be infected by *S. mansoni* as previous attempts to inhibit gene movement by use of a myosin inhibitor proved inconclusive due to the reversible nature of the drug this would not be an issue in the aged cohort. Alongside this fact is that it is widely accepted in the literature that it is more difficult to successfully infect adult snails as they exhibit increased resistance but resistance would wane with greater age (Richards and Minchella, 1987; Richards 1977). The aged cohort is significantly older than snails generally used in experimentation so it would be interesting to see if this increased resistance persisted. Half the aged cohort was also treated with remodelin, a HAT inhibitor, that as

shown in chapter 3 generally reduces resistance to infection either resulting in earlier maturation of the parasite in the snail with concomitant earlier shedding or increased average shed numbers. Remodelin is also used as a potential treatment for Hutchinson-Gilford Progeria Syndrome (HGPS) which is a partial progeriod syndrome in that it mimics several, but not all, the symptoms of advanced ageing so it would be interesting to see how this affected the aged cohort. As shown, all but one of the snails shed before they died and that one exception died early in the experiment so may not have been resistant, however the remodelin snails demonstrated better survival with one of the snails surviving to 6 weeks post infection and exhibited generally better survivability but not significantly so. This did show however, that the aged cohort was just as susceptible to infection as the juvenile cohort. Now whether this is a function of a decline in their immune response or whether gene movement in general is not as important a factor for progression of infection is unclear. Until further tests can be done in juvenile which actively inhibit NM1β only and see if that confers any resistance or a similar drug that puts a halt to gene movement, it does however open up the possibility of studying decline in innate immune response as a consequence of ageing if this can be shown to be the cause for this return of susceptibility in the aged cohort.

In developing this as a potential ageing model for genome organisation the next area to look at was general chromatin stability which was gauged by the accumulation of aneuploidy exhibiting nuclei. This showed a significant increase in the percentage of nuclei that had more copies of the *hsp70* gene loci than would appear in normal healthy cells. This accumulation of aneuploidy containing cells could be indicative of any number of factors from increased number of senescent cells which have become senescent due to potential for cancerous growth, to actual tumour growth or a function of decreasing fertility within the aged cohort. Any of these factors however show that *B. glabrata* could be promising for investigating ageing related accumulation of dysfunctional cells in relation to genome organisation.

It has been demonstrated that old snails exhibit an altered genome organisation, that changes with age with juvenile, 9 month and aged (12 month) snails all having different canonical positioning of *hsp70* loci. That in the aged snail there is a change in NM1β distribution with a potential decrease in protein expression, when comparing juvenile to aged snail nuclei. As well as an inability for known inducers of gene repositioning, heat shock and *S. mansoni* infection, to induce such gene repositioning in aged snails. These *in vivo* changes mimic those seen *in vitro* in human cells passaged into senescence (Bridger

unpublished data). With all these factors combined with the ease of husbandry and drug treatment, relatively short life span, similarity to human genome organisation and reduced ethical consideration allowing the *B. glabrata* model to come under Replacement for the NC3R's initiatives. Therefore, for the purposes of genome organisation relating to age *B. glabrata* will make an exemplary model with the potential to expand research into other age related disciplines of study.

Chapter 5: Discussion

5.1 Discussion

Schistosomiasis is a neglected tropical disease that is endemic throughout the tropics and sub-tropics with an estimated 250 million individuals infected and further 750 million living in endemic areas (Walz *et al.*, 2015; Colley *et al.*, 2014; Vos *et al.*, 2012). With population growth and raising global temperatures it would not be surprising for these numbers to continue to rise as there is currently no effective measure to eradicate this debilitating disease. Treatment is currently dependant on a single drug, praziquantel, however, there have been sporadic instances in the wild (Botros *et al.*, 2005), and lab induced (Giboda and Smith, 1997; Fallon *et al.*, 1996), of resistance. Attempts at developing a vaccine to the parasite are ongoing but, are progressing slowly due to limited success in stimulating the adaptive cellular immune response that is crucial for parasite clearance alongside the more typical humoral response. To this end it would be beneficial to develop a greater understanding of the mechanisms of infection used by the parasite to infect its host and in investigating this a potential new way to interrupt the progression of *S. mansoni* life cycle has been discovered. Sodium butyrate, a class I HDAC inhibitor currently available as a supplement, has exhibited the ability to confer greater resistance to exposed snails.

5.1.1 Drug inhibition of infection

Histone acetylation modifications tend to be quicker to change, with higher turnovers (Waterborg, 2002), although some acetyl sites have been shown to be more stable (Zheng, Thomas and Kelleher, 2013). Therefore histone acetylation are potential targets for the early changes seen in the host resulting in movement of genes within 15 minutes of infection (Arican-Goktas *et al.*, 2014). As such drugs that interfere with histone acetylation, HAT and HDAC inhibitors, were a viable target to try and affect the early changes during infection. Because of this 3 drugs targeting histone acetylation, two HAT inhibitors and one HDAC class 1 inhibitor, sodium butyrate, were used. These established a clear role for histone acetylation in *S. mansoni* infection of *B. glabrata*. Although the effects of the two HAT inhibitors were less pronounced resulting in earlier shedding and / or occasionally greater cercariae numbers in early weeks than controls. This highlighted the effects of the HDAC which resulted in 100% resistance in snails that survived the trial. While the survival rate from sodium butyrate exposure was comparatively poor the results were clear. Although not the first drug to inhibit infection in *B.glabrata* discovered as other compounds, like the

HSP90 inhibitor geldanamycin has previously been shown confer resistance in the snail (Ittiprasert and Knight, 2012).

One advantage however, is that sodium butyrate is currently sold as supplement so could be made comparatively cheap. Therefore if the effects seen *in vivo* in *B. glabrata* could be repeated *in vitro* in human cell and cercariae co-cultures it would be easier to set up trials. In fact sodium butyrate has already be tested as an adjunct therapy for othe infectious diseases (Raqib *et al*, 2012). This means that it could also be used as potential treatment on its own or alongside praziquantel or if not effective in humans used alongside or in replacement of molluscides like *Euphorbia milii* latex (Augusto *et al*, 2017). Sodium butyrate is water soluble meaning it may be possible to use it to treat water systems if the minimum effect dose is researched and found to be viable. If the environmental impact of adding it to the water could also be examined it may prove to be effective at limiting the spread of schistosomiasis without impacting on the ecology of the snails. Furthermore even if it is not viable as a treatment option it has narrowed down important changes that are required by *S. mansoni* and could help narrow research for vaccine development. This would be via helping narrow the targets for development to the component or components of the ESPs which are demonstrated to affect acetylation in the host.

5.1.2 Establishing an ageing model

With the effect which HDAC and HAT inhibitors have when the host is treated it is evident that *S. mansoni* caused alterations to the epigenetics of its host *B. glabrata*. This is congruent with changes to genome organisations described in snails susceptible to infection where the movement of certain gene loci are observed. It has also been observed that with snail strains vulnerable to infection there can be varying levels of susceptibility throughout their lifespan with juvenile and old snails generally being more susceptible than egg laying adults (Richards and Minchella, 1987; Richards 1977). This could indicate alterations to genome plasticity or organisation between juvenile and adult and old snails that impacted susceptibility. This indicated that there were possibly alterations occurring to the epigenome of the snail as it developed which hampered the action of the parasite to control its host.

The genome organisation of the interphase nuclei of *B. glabrata* is similar to that of human nuclei and *in vitro* experiments on senescent cells had demonstrated that the ability to move genes or chromosomes in the interphase nuclei is lost (Bridger, unpublished data). Since gene movement (Arican-Goktas *et al,* 2014) and alteration to genome organisation (Odoemelam, 2009) is affected by the parasite the effects of age on genome plasticity in the snail were investigated. These experiments on 12 month old snails exhibited remarkable similarity to what is seen in *in vitro* human senescent cells. Gene position was significantly altered and the gene did not move when exposed to either heat shock or parasite infection. With this it was demonstrated that *B. glabrata* was a viable model for investigating the effects of ageing in a living organism with similar genome organisation to that of human nuclei. It also demonstrated that gene movement in these older snails did not occur. However, the aged snails did not prove any more difficult to infect than the juvenile usually used with all infected aged snails exhibiting active shedding.

Unfortunately the cessation of gene movement in the aged cohort did not prove enough to provide resistance to infection. However, as with all aged creatures there may be other factors involved that could render them more susceptible to infection. It was demonstrated that the aged snails had significantly more nuclei exhibiting *hsp70* aneuploidy than juvenile snails which would point to a loss of regulation and clearance of such cells which could be due to a declining immune system. This could indicate that although gene movement cannot occur in the weakened state of the aged snail it simply is not necessary for parasite infection. Other attempts at preventing gene movement using BDM proved inconclusive as the drug was reversible within a relatively short time frame and gene movements were merely delayed rather than completely prevented. The target in this case were the nuclear myosins, for their role in genome plasticity to be fully explored and importance of gene movement to *S. mansoni* infection it will be necessary to develop siRNA inhibitors specifically targeted against the nuclear myosins. However, as with senescent human cells *in vitro* it was demonstrated that the nuclear myosin 1β showed altered pattern distributions in the aged snail cells further supporting the aged snail as a viable model for investigating ageing *in vivo*.

5.1.3 Epigenetic modifications affected by stress

Alterations in genome organisation, like those exhibited between juvenile, 9 month old and aged 12 month old snails are probably indicated for by shifts in long term epigenetic

modifications, which would suggest changes to histone or DNA methylation states. Experiments focused on changes brought about by heat stress in snails as this replicated the gene movement observed in infection. This would allow for the assessment of any global changes in histone modification pattern distribution that could be detected due to age but also changes in stress response capabilities as an organism aged as well. Modifications known to increase with age and gene silencing, H3K27me^3 (Ma *et al.*, 2018; Dou *et al.*, 2017, Wiles and Selker 2017) and H4K20me^3 (Evertts *et al.*, 2013) as well as a modification associated with gene activation (Nguyen and Zhang, 2011) and linked to *S. mansoni* infection H3K79me^3 were selected.

H3K27me^3 showed significant changes as a result of heat shock only within the juvenile snail cohorts. However, the marked reduction seen in H3K27me^3 punctate staining in juveniles resulted in the juvenile punctate staining post heat shock decreasing to the level that was closer to both the adult (3 month) and aged (12 month) cohorts in either their unstressed or heat shocked state. Although there is a significant difference between the aged heat shock and juvenile heat shock but not between heat shocked juvenile and adult or adult and aged cohorts. The major difference in response to ageing and heat shock was seen in the distribution of the remaining patterns. Peripheral staining increased in response to heat shock in both juvenile and adult, with adult snails exhibiting more peripheral patterning to begin with. Whereas this peripheral stain has declined with age and in response to heat shock actually decreases further. Directional patterning also increases after heat shock in juveniles although remains unaffected in both adult and aged cohorts the pattern distribution differs with adult cohorts having number similar to juvenile post heat shock in both controls and heat shock whereas aged cohorts controls and heat shock show patterning distribution to control juvenile. The decrease in peripheral staining in the aged cohort could be a result of the formation of CCFs which are known to contain H3K27me^3 modified chromatin (Dou *et al.*, 2017). However, the most striking difference is in the appearance of a new pattern in the aged cohort, speckles.

A similar shift was seen in both H3K79me^3 and H4K20me^3 with both exhibiting decreases in the more evenly distributed punctate pattern to more specialised patterns with age, although the changes in the H3K79me^3 were only really evident in the aged cohort or after heat shock in adult or aged snails. While H4K20me^3 exhibited a shift in pattern distribution from juvenile to adult, the distribution did not significantly change for between adult and aged except for the appearance of new patterns. Although fewer in number than those of the new pattern in

H3K27me^3 aged samples, this could indicate a loss of regulatory control as histones are being modified which either should not be or genome plasticity has decreased not permitting the rearrangement to the correct patterns to occur. Of interest is the H4K20me^3 modification as not only does it show significant change from juvenile to adult but, after heat stress, aged cohorts exhibit the opposite response from juvenile and adult, with the aged cohort demonstrating increased speckle staining and juvenile and adult cohorts a decrease after stress. In fact the change in speckle staining happened in all three modifications and exhibited similar percentage distribution in aged pre and post heat shock. This likely supports a loss of genome plasticity as modifications were being made but redistribution wasn't occurring, potentially as a result to a change to the NM1β distribution (Mehta *et al* 2010).

5.1.4 Epigenetics affected by infection

Having shown significant changes to histone modification patterning throughout life comparisons were made to juveniles snails post infection to assess what changes the parasite induced. H3K27me^3 which had exhibited such significant changes with age showed comparatively little change between control and 2h post infection, the patterns mimicking closely that seen in heat shock. H4K20me^3 conversely showed a divergence from the juvenile heat shock model and instead mimicking the response to heat shock seen in the aged cohort study. Although the increase in speckles was not significant for infection the decrease in peripheral was and though not as dramatic as that seen in heat shocked aged snails the general shift in patterning was the same.

H3K79me^3 was the most dramatic change as seen in chapter 2 the shift in patterning was unique to infection by the parasite and completely separate from that caused by heat shock model that replicated the loci movement. As such this modification would be a focus for further study, however, optimising ChIP qPCR can be difficult in a new model organism and although histone modifications are quite well conserved throughout organisms there is no guarantee an antibody will work and if it does for IF and is certified ChIP ready there is no assurance that it will work for the ChIP application (Luu *et al*, 2011). Similarly optimising ChIP qPCR requires knowing roughly where the histones may be found within the gene sequence to properly target the primers. As such a method that would allow for better visualisation of the co-localisation of gene sequence and histone modification could act as

linking experiment between IF and ChIP qPCR. For this the immuno-fibre-FISH on *B. glabrata* protocol was optimised. With the goal of allowing better targeting of gene and histone modification associations and potentially allow for quantitative analysis of images in the case the antibodies for IF work but ChIP fails.

With the optimised fibre-FISH protocol the screening of more histone modifications can be done to narrow down specific targets that may be associated with parasite induced gene movement. These could also prove useful for not just understanding the movement induced due to parasite infection but, what modifications are involved in gene movement in general. From this understanding of what histone modifications are involved in signalling gene movement, or if specific codes of histones act as guiding coordinates to nuclear machinery that causes the rearrangement it may be possible to identify precisely what protein complexes are involved in rapid chromatin relocation. Furthering our understanding of what is required to relocate genes within the interphase nuclei could potentially indicate what signalling pathways *S. mansoni* needs to activate to cause the desired result. This would likely be a component of the ESPs the parasite releases upon infection of the host and may differ from that which causes change to the host acetylome. Once that component has been isolated it may be possible to ascertain if the protein has the same effect in the human host it will offer yet another target for vaccine development.

5.1.5 Further work

Having established changes to the pattern of histone modifications occurred during infection and that inhibition of changes to the acetylome affected snail resistance to infection the next step would be to investigate similar inhibitors to epigenetic modifications. Using the methods developed in chapter 3 for running drug trials within *B. glabrata* you could screen multiple histone modification inhibitors for their effectiveness against the parasite. Although the key would be to ensure that the drug was affecting the host's epigenome and not the parasite as EZH2 inhibitors have been shown to prevent the miracidia from being able to progress in their life cycle into sporocysts (Roquis *et al*, 2018). However, a drug that would be of particular interest would be pinometostat as this would prevent alterations to the methylation state of H3K79 which was shown to be affected by parasite particularly dramatically and in a way that was not replicated by heat stress or ageing.

The effect of NM1β inhibition needs to be further explored. The drug BDM had too many off target effects and ultimately due to its reversible nature did not inhibit the action of NM1β long enough to discover if this would have affected resistance to infection. This was most obvious with the 0.1M concentration that caused the snail to be paralysed for 30 to 45 minutes after exposure but regained movement within the hour. Since BDM affects all myosins this could be used as a proxy for how long drug remained in effect. Combine this with the gene loci position being different from both uninfected and untreated infected at 2h and there is no clear picture of what effect NM1β inhibition would have. To further explore this the use of siRNA to targetedly inhibit NM1β in *B. glabrata* would be the most efficient method. A protocol has previously been developed using PEI transfection that had been shown to be effective in *B. glabrata* (Knight *et al*, 2011b). Using this method it should be possible to acertain whether NM1β is directly involved in gene relocation and if preventing its action can confer resistance to infection.

Having developed the protocol for fibre-FISH the next step would be to establish if the *hsp70* loci in *B. glabrata* was associated with the H3K79me^3 modification prior to and / or after infection. Of the modifications investigated this is the only one that was uniquely linked to infection. It has also been previously shown to be difficult to evaluate using ChIP (Barski *et al.*, 2007, Steger *et al.*, 2008) in human cells. This combined with the potential issues using antibodies in a new model organism means that actually visualising the association in one state or another would be appropriate prior to taking the next step and using ChIP-qPCR to quantify the association. Ultimately using this method will eliminate some of the troubleshooting involved in ChIP work looking at targeted gene sequences by at least confirming association.

In relation to the aged snail model there are a couple of experiments that could improve the model itself. These would include the development of chromosome paints and assessing chromosome territories to identify if it is single gene loci moving or whole chromosome as in the human cells *in vitro* (Mehta, 2010). As for using the model, investigating alterations to gene position at more age points to assess if it is a gradual change with age for if there are milestones where the majority of these alterations occur would be interesting. Simialrly investigating the effects of certain drug treatments such as resveralogues (Latorre, 2017) or the effect of periodic stressing such as heat shock or cold shock could afford new insights into what affects the ageing process and how it can be modulated.

5.1.6 Conclusion

Within this thesis it has been demonstrated that the epigenome of the *B. glabrata* snail is significantly affected by *S. mansoni* infection, heat-shock and ageing. As such I have even identified a specific marker, H3K79me^3, which may be uniquely affected by *S. mansoni* during infection. Tools have been developed to further elaborate how those changes relate to target genes that are known to move during infection, which can also aid in troubleshooting for ChIP and antibody issues. Similarly it has been demonstrated that directly interfering with certain histone modification changes within the host can affect *B. glabrata* susceptibility to infection by *S. mansoni*. That by modulating the hosts' ability to either add or remove actyl groups to histones their susceptibility to infection either increases or decreases. It has also been exhibited that although adult snails in the literature are more resistant to infection aged snails are no harder to infect than the sexually immature juveniles. However, in investigating this fact *B. glabrata* has come to the fore as a new model for investigating the effects of ageing *in vivo* in an organism with similar interphase chromatin organisation as humans.

In closing, this research presents new tools for investigating the effects host:pathogen dynamic on chromatin organisation of the host. Identifies modifications elicited in *B. glabrata* by *S. mansoni* to support its own survival. Shows that by interfering with *B. glabrata* ability to modulate its chromatin organisation directly affects *S. mansoni* survival as it likely uses those same mechanisms to affect change for its own survival. As well as establishes *B. glabrata* as a new model organism for investing the effects of ageing on chromatin organisation.

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