Chapter 6

Concluding remarks

Sexual maturation of female schistosomes is critically dependant on stimulation by a male partner, as is well established (Basch, 1991). In the absence of a male stimulus, the female worms remain developmentally stunted without mature ovaries and vitellaria on which egg production depends. While the differences between fully mature females and immature females had already been explored extensively [for example Fitzpatrick & Hoffmann (2006) and Waisberg *et al.* (2007)] when I started working on this thesis, a time course of gene expression, measured using RNA-Seq, over the course of female development was not available. This time course revealed the changes in gene expression as they occurred over time after pairing commenced, rather than just the gene expression of fully mature females. Many studies have examined the signalling cascade that leads to the proliferation of cells in the female reproductive organs (see Chapter 1.5) and there was already evidence for the involvement of neuropeptides (Collins *et al.*, 2010) as well as apoptosis (Galanti *et al.*, 2012) playing an important role in female development, and particularly regulating cell proliferation in female reproductive organs.

Recently, several papers examined the changes in genes expression over the course of schistosome maturation (Cai *et al.*, 2016; Wang *et al.*, 2017). One of their aims was to discover the precise stimulus used by male worms to induce maturation in females. The nature of this stimulus has been the subject to research and speculation of years, but has remained elusive (Chapter 1.4). Wang *et al.* (2017) used their expression data to identify genes whose expression was correlated with paring or vitelline development. Wang *et al.* (2017) hypothesised that an aromatic-L-amino acid decarboxylase (AADC) gene, which they showed

to be expressed in the gynecophoral canal of male worms using WISH, was responsible for the stimulation of female worms. The authors did not present RNAi evidence showing that knock down of AADC prevented female maturation as they did for another gene in the same paper. Thus, the evidence for AADC inducing female fertility seems somewhat circumstantial at this point, as it appears to be based on a correlation of AADC expression with female fertility, as well as the expression in the gynecophoral canal. Furthermore, data from Chapter 3 showed that the *S. mansoni* homologue of AADC is expressed in a paring-dependant manner. However, that would raise the question of how male worms are stimulated to express AADC. More concrete evidence, such as RNAi experiments, similar to that provided for the allatostatin receptor in the same publication (Wang *et al*., 2017), would greatly strengthen this hypothesis.

This thesis examined the events leading to maturation of female worms starting with pairing and the male-female interaction. This is an important aspect of schistosome biology for many reasons. In particular, if untreated, adult schistosomes survive for years inside the mammalian host, and their egg production is responsible for much of the pathology associated with schistosomiasis as well as its transmission. Hopefully the information gathered here can help to identify new avenues in the search for novel drug targets and therapies. Praziquantel, the current drug of choice for the treatment of schistosomasis, is not effective against developing parasites and also faces the challenge of emerging resistance (Crellen *et al.*, 2016).

Divergent development of males and females

In Chapter 3, the development of male and female worms was examined at the transcriptome level, to determine when changes in gene expression take place and how they differ in worms from mixed sex and single sex infections. Male and female *S. mansoni* are still highly similar at the transcriptome level at 18 d.p.i. with only 38 DEGs identified between them. As pairing commences, their differences increase to 173 DEGs at 28 d.p.i. and continues to 2194 DEGs at 35 d.p.i. when sexual maturation of the female worms has significantly progressed. Both male and female worms expressed genes involved in the cytochrome P450-dependent drug metabolism pathway at higher levels at 18 d.p.i. than at later time points. Of these genes, glutathione S transferases (including Smp_024010 and Smp_102070) in particular are thought to play a key role in the mediation of xenobiotic metabolism in schistosomes, and have been shown to bind praziquantel at a non-substrate site (Cardoso *et al.*, 2003). Their high expression levels at the earliest point during the time course suggests that these glutathione S transferases may be responsible for the resistance of immature worms to praziquantel. Expression of genes involved in spermatocyte production such as the testes expressed transcription factors *boule* (Smp_144860) and basonuclin 2 (Smp_138350) was found to change from 18 d.p.i. onwards in male worms. The expression of boule, which in *Schmidtea mediteranea* is responsible for meiotic progression of male germ cells, rose significantly over the following time points. In contrast, the expression of basonuclin 2, which prevents premature maturation of spermatocytes in mammals, was found to decline significantly after 18 d.p.i.

Relatively few differences were found between males of mixed sex and single sex infections during their development. A notable exception to this was an aromatic-L-amino acid decarboxylase (AADC) described by Wang *et al.* (2017) (see above). Female worms are well known to up-regulate fertility related genes such as eggshell proteins and tyrosinases once paired, and this was observed consistently from 28 to 38 d.p.i. in mixed sex infections. A large number of transcription factors became down-regulated around the time pairing commences in females from both mixed sex as well as single sex infections. This is interesting because it showed that the down-regulation occurs in a pairing-independent fashion (Chapter 3.2.5). After an approximately three week period of maturation the female worms become developmentally arrested, which coincides with this down-regulation of transcription factors. Only after pairing, three zinc-finger transcription factors (Smp_166560, Smp_087320 and Smp_095350) became up-regulated. Their homologues in *D. melanogaster* regulate expression of neuropeptides (Vogler & Urban, 2008) and the response to stimuli such as growth factors (Baou *et al.*, 2009). If this function were conserved in schistosomes it would potentially allow them to regulate the development of female schistosomes to reach sexual maturity.

In vitro culture has significant effects on neural genes

Having examined the process of maturation in Chapter 3, Chapter 4 aimed to examine the opposite: regression of female worms in the absence of a male partner. However, when comparing RNA-Seq data of paired and single worm, it became apparent that *in vitro* culture has an extensive effect on gene expression. Changes due to *in vitro* culture, especially of fertility related genes, had been

expected in female worms as reported in the literature (Galanti *et al.*, 2012). However the changes in gene expression measured here were neither limited to fertility related genes nor to female worms. Using RNA-Seq the whole transcriptome could be examined rather than being limited to small numbers of genes as in previous studies. As a result a large group of neural genes involved in regulating the development of the nervous system was found to be up-regulated *in vitro*. These genes, such as semaphorins and their plexin and neuropilin receptors, netrins and the DCC and UNC5 receptors, slit and its robo receptors as well as ephrin and its receptors, have all been implicated in the pruning of axons and controlled neural cell death (Vanderhaeghen & Cheng, 2010). On the other hand, neuron survival genes, such as those coding for motor neuron 1 and fragile X mental retardation protein 1, were found to be down-regulated. Together these expression results suggested that parts of the worm nervous system regressed together with the reproductive tissues. This may be of particular interest as the nervous system is thought to be involved in the regulation of female fertility (Collins *et al.*, 2010; Wang *et al.*, 2017).

The role of CD63 receptor and antigen in female fertility

The analysis of RNA-Seq data revealed the up-regulation of two tetraspanins in females from mixed sex infections from 28 d.p.i.: CD63 receptor, previously reported by Fitzpatrick *et al.* (2009), and CD63 antigen. Using RNA-Seq and qRT-PCR data, mature paired females were shown to express the CD63 receptor and antigen at 800- and 50-fold higher levels than male worms as well as at over 190- and 39-fold higher levels than females from single sex infections. Also, both genes were found to be depleted in the ovaries of paired females, compared to

whole worms and the *in situ* experiments showed expression predominantly in the vitellarian tissue. Furthermore, in Chapter 3, both genes clustered with several well-known fertility related genes strongly suggesting a role in the development or maintenance of female fertility. RNAi provided some evidence of a RNA knock down affecting egg production. However, the RNAi experiment was performed *in vitro* and was therefore confounded by the associated problems of reduced fertility in the negative control. Female fertility is particular difficult to study *in vitro*, as regression of female reproductive tissues occurs quickly outside the host although Galanti *et al.* (2012) showed that this process is slowed in the presence of male worms. Although only a moderate reduction of expression was achieved for the CD63 antigen and no significant reduction for the CD63 receptor, both knockdowns were found to result in a significantly reduced production of eggs (CD63 antigen: 9.6-fold; CR63 receptor: 3.2-fold), compared to the negative control.

Given this evidence, a role of both CD63 receptor and antigen in egg production seems near certain. However, their exact function in this process is unclear. In humans the CD63 receptor is involved in forming signalling complexes of integrins and growth factors and thereby regulating downstream gene expression and cell proliferation. If the schistosome homologues performed similar functions, such a role could be crucial to regulate the development of vitellarian tissue in paired female worms. Furthermore, both the CD63 receptor and antigen have been implicated in the regulation of cell survival by down-regulating integrin-β signalling and consequently down-regulating apoptosis in human cell lines. Up-regulation of these two genes in paired females could allow

for proliferation of vitellocytes by reducing the rate of apoptosis in this tissue. The CD63 receptor has also been associated with intracellular vesicles and regulation of protein transport in and out of such vesicles. During their maturation vitellocytes become filled with intracellular vesicles in which precursors of the egg shell proteins are stored. This could mean that CD63 receptors play a role in the formation of such vesicles.

To further explore the role of CD63 receptor and antigen it would be useful to study their interactions with other proteins or maybe even with one another. This might be possible by using a yeast two-hybrid assay which has been used identify other protein interactions in *S. mansoni* (Vanderstraete et al., 2014). Anotherpossible method might be the use of fluorescence resonance energy transfer to observe co-localisation of CD63R and CD63a, however this would require the availablitliy of anitbodies with binding specificity to the schistosome genes. A similar approach has been used successfully before to study the interaction of tetraspanins with other proteins (Termini and Gillette, 2017).

Apoptosis in *S. mansoni*

Finally, in Chapter 5, gene expression in the gonads of worms from mixed and single sex infections was examined. This enabled a set of genes to be produced with ovary- and testes-biased expression and the effect of pairing in the worm gonads to be analysed, which had previously been obscured by the somatic tissue. Several apoptosis genes have been identified in schistosomes previously and apoptosis has been shown to play an important role in regulating the growth of vitellarian tissue. In Chapter 2, many genes were examined for their sequence

homology and the presence of appropriate domains in particular, to expand the list of schistosome apoptosis genes. Among the 15 identified genes were homologues of the FAS associated factors 1 and 2, a homologue of the tumour necrosis factor receptor as well as two Bax inhibitors, adding considerable detail to how apoptosis potentially functions in *S. mansoni*. Having divided these genes in to pro- and anti-apoptotic genes based on the reported functions of orthologs, the expression of such genes was observed in several experiments. No consistent pattern of apoptosis related genes was found *in vitro* (see Chapter 4). However, a significant down-regulation of putative pro-apoptotic genes and, vice versa, an up-regulation of putative anti-apoptotic genes was observed in the ovaries of females from mixed sex infections, compared to ovaries from single sex infections (see Figure 5.17). This further strengthened my confidence that these genes are involved in the regulation of apoptosis in *S. mansoni*.

The limitations of bulk RNAseq

RNA-Seq is a powerful method to study gene expression, making it possible to measure changes in mRNA levels for all genes at once. And as discussed in the introduction (see Chapter 1.6.3), RNA-Seq offers some significant advantages over other methods used to measure gene expression. However, there are also some limitations to RNA-Seq as used in this thesis. For one, studies by Schwanhaeusser *et al.* (2011) and Vogel *et al.* (2010) found the steady-state mRNA concentrations to only explain between one and two thirds of the variation in observed protein levels. However, work by Li *et al.* (2014) showed that due to problems with the normalisation of proteomics data in the aforementioned studies, mRNA may explain around 84% of protein abundances.

Furthermore, the correlation of mRNA and protein abundance were shown to correlate strongly when specifically looking at changes in gene expression, meaning that the mRNA abundance of differentially expressed genes is in fact very well correlated to their protein products (Koussounadis *et al.* 2015).

Another limitation is the use of RNA-Seq on mRNA extracted from whole worms, rather than individual organs or even single cells, although this was done to some extent in Chapter 5 of this thesis. When examining genes that are only or predominantly expressed in a subset of organs, changes in gene expression may be more difficult to detect. This is because in a sample derived from a whole worm, the mRNA of such organ-specific genes will make up a lower proportion of total reads. Another variation of this problem occurs when comparing samples from different developmental stages. As organs such as the vitellarium develop, they start to make up an increasingly large share of the sequenced mRNA. Due to this, it may appear that certain genes in these female worms have become down-regulated, when in reality the absolute expression has not changed but the gene's mRNA now makes up a relatively smaller proportion of the total mRNA pool. For instance in Chapter 3.2.8, the reads from just 27 genes, most of which are known to be expressed in vitellarian cells, made up >14% of the transcriptome of mature females. As a result many other genes appeared to be expressed at lower levels in mature females compared to females from single sex infections. Lastly, when performing RNA-Seq on whole worms, in essence the average gene expression across all tissues is measured, despite there potentially being considerable variation in expression in organs or even adjacent cells. In such samples the average of gene expression in all tissues may show a different

qualitative trend than when all tissues had been measured individually, an effect known as Simpson's paradox (Trapnell *et al.*, 2014). Although RNA-Seq data from different tissues or even single cells would provide an even better and set of data with greater resolution of schistosome transcriptome, RNA-Seq data derived from whole worms still holds a wealth of information. Arguably, for genes expression in a single tissue, such as many genes expressed in the reproductive organs, there must be a very strong change in expression for it to be detected as significantly expressed.

As the above summary of results shows, the work in this thesis succeeded in generating high quality RNA-Seq data that illuminate the changes in gene expression that result in female maturation, following their development from approximately one week before pairing occurs to sexual maturity. The data generated here also covered transcriptome changes *in vitro* as well as gene expression in the gonads of male and female worms. The analysis presented in this thesis also showed numerous examples of differences between male and female schistosomes during their development as well as the changes induced by the male-female interaction.

In the future, the use of single cell RNA sequencing (scRNA-seq) could address some of the shortcomings of bulk RNA-seq. Most importantly, it would provide a finer resolution of gene expression, not providing transcriptome data of whole worms or individual organs but of single cells. With this technology, it could be possible to detect and quantify the abundance of mRNAs coding for the CD63 receptor and antigen in different subpopulations of vitellocytes. The vitelline

tissue could be dissociated using a collagenases or dispases (Freshney, 1987) and fluorescence activated cell sorting (FACS) could be used to sort cells by their granularity (Basu *et al.*, 2010). This may allow vitelline S1, S2, S3 and S4 cells to be separated as they contain increasing numbers of vesicles (Erasmus, 1975), with S4 cells being most densely packed with eggshell protein containing vesicle (Köster *et al.*, 1988). With this method the localisation of CD63R to particular portion of the vitellaria could potentially be confirmed. Also, very small subpopulations of cells could potentially be examined, such as stem cells, which have previously been observed (Collins *et al.*, 2013), and could play a role in the ability of *S. mansoni* to regenerate reproductive tissues after losing it. Similarly, scRNA-seq could provide unprecedented resolution of the development of cells in the ovary and testes. This may make it possible to better understand the role of apoptosis in the development of ovarian tissue.

However, not all of the goals of this thesis could be met. In particular, examining the regression of sexually mature females as a result of separation from male worms in Chapter 4 proved challenging. Despite several biological replicates, the number of differentially expressed genes found between treatment groups was low. This most likely resulted from the large magnitude of transcriptome changes brought on by *in vitro* culture, which was shown to have extensive effects on the transcriptome of male and female worms. Due to this, the focus of the chapter changed to examining the effects of *in vitro* culture on gene expression, especially neural and apoptosis-related genes. The transcriptome changes observed in Chapter 4, most likely brought on by a lack of host factors, highlights the need for a better *in vitro* culturing system. Alternatively, worms

could have been re-implanted into mice (Basch & Humbert, 1981) after separation from their partner, to provide a host environment for the rest of the experiment. However, this method would have required more mice to be subjected to invasive procedures, moreover it would expose the worms themselves to considerable stress, which might have interfered with gene expression. Another improvement could have been to perform the RNAi experiment described in Chapter 3 *in vivo*, as described by (Wang *et al.*, 2017). As described above, the *in vitro* conditions caused changes in gene expression that made it difficult to detect the changes in gene expression brought on by the dsRNA treatment compared to the control. Performing RNAi *in vivo* circumvents that problem by making *in vitro* culture during or after exposure to dsRNA unnecessary.

The results in this thesis, including a better understanding of the male-female interaction as well as how apoptosis functions in schistosomes, will hopefully prove valuable for the development of therapeutics in the future. For example, in addition to GST28 (Smp_054160), a long-standing vaccine target, other glutathione S transferases, such as Smp_024010, may also be suitable therapeutic targets. Also an improved understanding of how schistosomes regulate apoptosis could make it possible to exploit differences between schistosome and mammalian apoptosis to kill worms. The data generated for Chapter 5, in collaboration with Professor Grevelding's group, has also been made available to the research community as a resource for gene expression data in the gonads of adult male and female worms (Lu *et al.*, 2017).

To further investigate apoptosis in the ovaries, but also the vitelline tissue, a number of approached could be taken in the future.

To determine how much apoptosis is taking place in the ovaries of females from MS and SS infections, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining could be used, as Galanti *et al.* (2012) did to detect apoptosis in the vitelline tissue. TUNEL staining is a method that detects the DNA fragmentation that occurs during apoptosis (Kyrylkova *et al.*, 2012). This is done by using the terminal deoxynucleotididyl transferase to conjugate labelled nucleotides, for example with a bromodeoxyuridine (BrdU) label. In the next step, these nucleotides can then be detected with a method appropriate for the chosen label (Kyrylkova *et al.*, 2012). In the case of BrdU, this can be done with antibodies with conjugated alkaline phosphatase, allowing BrdU detection using a method like that used in WISH (Chapter 2.2.7). Another method which could be used to study apoptosis is an assay for caspase 3 activity (Galanti *et al.*, 2012).

In Chapter 5, differences in the expression of gene coding for proteins of the sphingolipid metabolite pathway were observed in the ovaries of females of SS and MS infections. Two important enzymes regulating the abundance of ceramide in cells are ceramide synthase and ceramidase (Levy & Futerman, 2010; Mullen *et al.*, 2012). Inhibitors of these enzymes could be used to investigate if these enzymes also play a role in regulating apoptosis in *S. mansoni*, as they do in mammalian cells (Separovic *et al.*, 2012; Zhu *et al.*, 2008). Fumonisin B, a fungal toxin, has been shown to inhibit ceramide synthase and reduce apoptosis in human cells (Boppana *et al.*, 2014), whereas carmofur is a

potent ceramidase inhibitor (Realini *et al.*, 2013). Using TUNEL staining, it may be possible to examine, if an effect on apoptosis can be observed in females from SS and MS infections when exposed to these inhibitors. Treatment of females from SS infections may prevent apoptosis of vitellarian cells and allow maturation of the organ. On the other hand, treatment of females from MS infections with carmofur, may result in higher levels of apoptosis and lead to a shrinkage of the vitellarian tissue.

There are also fluorescence-based assays available to measure the activity of ceramide synthase (Kim *et al.*, 2012) and ceramidase (Bedia *et al.*, 2010), which could be used to confirm the specificity of the inhibitors to the schistosome homologues of these genes.

By addressing the limitations discussed above, new insights can be gained. Single cell RNA-Seq especially will open up new avenues for characterising gene expression at much greater resolution and improve our understanding of schistosome biology. Secondly improving the available *in vitro* culture methods will allow for better characterisation of genes, which may become targets for future interventions.