# Enhancing Production:

# Effects of Royalactin on

# Insect Development

**Thesis submitted for the degree of Doctor of Philosophy**

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# Abstract

Phenotypic plasticity is a phenomenon found in eusocial insects that produces distinct female castes through phenotypic responses to environmental change. Honey bees (*Apis mellifera*) are a perfect model to study phenotypic plasticity. Worker bees and queen bees, both of which are female, have different adult phenotypes, depending on the diet the larvae are fed during development. Contained within this diet, known as royal jelly, is a protein called 'Royalactin'. An earlier study showed that Royalactin can induce a queen-like phenotype in female bee larvae and promotes growth and life-span in other species, such as the fruit fly *D. melanogaster* and the nematode *C. elegans*. An initial aim of this study was to produce Royalactin in an *E. coli* expression system and then test its potential growth effects in a set of insects of economic importance. The recombinant protein was produced using an *E. coli* expression system and confirmed by amino acid sequencing. This thesis describes alternative methods to obtain body-enlarging effects in insects. DNA-methylation inhibitors were used to increase body weight, which prompted the use of RNA interference to study in detail what genes are involved in body growth during development.

The results suggest that insect body weight can be modified during development by interfering with the DNA methylation system. More work needs to be carried out, using RNA interference, to pinpoint the genes involved in the process.

# Acknowledgements

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# Chapter 1 Introduction

This project was conceived as part of a collaboration between the University of Nottingham and Monkfield Nutrition Ltd., a company that produces insects as animal feed. This partnership was created to investigate the possibility to modify the phenotype of some of the insects that Monkfield Nutrition Ltd. produces so that their insect production would benefit from these alterations.

During the years Monkfield Nutrition Ltd. expanded their market from providing insects as pet food, to producing insect flour as feed supplement, with the future possibility to be usable for human consumption. It is in this context that an increase in body size, a longer lifespan, and a higher fertility are all commercial interests for Monkfield Nutrition Ltd. and all possible improvements on the creation of a better final product.

Feeding insects to humans is something that is shaping to become an important resource in future years, with the need to produce more food for an increasing world population, but the need to reduce emissions and land exploitation. Animal and plant breeding have been a constant priority for humankind to modify them to better suit them to our needs, insects, conversely, have been ignored for a long time and only recently they are starting to be seen as a food resource ( van Huis et al., 2015; Ordoñez-Araque and Egas-Montenegro, 2021). Insects are also important in global food security as they can also help tackle the reduction of resource depletion due to their smaller need of feed to grow compared to the more traditional endothermic livestock (birds and mammals) and the reduced need of space (Hawkey et al., 2021).Insects nutritional

composition varies from species to species, but models showed how they could help tackle some nutrition-related public health problems (Payne et al., 2016) and do so being a low-cost solution making it accessible to everyone (Carvalho et al., 2020). Yet the biggest challenge in the western world is to create an inviting image of insects to reduce the stigma of eating them (Bessa et al., 2020).

This thesis is unified by the core theme of generating new insect phenotypes by modifying the organisms during their development; the phenotype that I focused on was increased body weight. The idea of increasing the body weight of insects was obtained from a study by Kamakura (2011) in which it was reported that it is a single component of royal jelly, a protein molecule themed Royalactin that triggers the development of queen bees. Kamakura tested the effect of Royalactin by adding it to the diet of honey bees (*Apis mellifera*) and fruit fly (*Drosophila*) larvae and obtained adult queens, and adult individuals with enlarged abdomens and increased body size, respectively. These effects could be beneficial in creating a higher quality product that could help create a better solution to the global food security issue that I introduced earlier.

The initial aim of this thesis was to replicate the body-enlarging effect on some of the insect species produced by Monkfield Nutrition Ltd. by adding the same component used by Kamakura to the insects' diet. Subsequently, the means to obtain this effect changed to focussing on using chemicals that were shown to mimic the effects of the protein and, ultimately, to silencing genes that are either targets of the pathways activated by the protein or involved in the development of insects. Experiments were performed mainly on to two insect species: the parasitoid wasp *Goniozus legneri* Gordh

(section 1.5.1) and the cricket *Gryllus bimaculatus* De Geer (section 1.5.2). One of the insects that I used in this project is the parasitoid wasp *Goniozus legneri*, females of this wasp species lay eggs on several species of caterpillars. These caterpillars are considered pests as they cause pre-harvest damage to commercially plants or post-harvest damage to stored grains, flour or dried fruits. *Goniozus legneri* is used as a biocontrol agent to suppress pest populations. In this case increasing insects' size can have positive effects on the efficacy of biocontrol: Hardy et al. (1992) shows that larger individuals of the parasitoid wasp *Goniozus nephantidis* (which has very similar life cycle to *G. legneri*) live longer and have higher fecundity, while Petersen and Hardy (1996) show that they also compete better for essential resources. Goubault et al. (2006) shows that *Goniozus legneri* do better in fights when they are larger. And finally, O'Neill and Skinner (1990) show that there is a positive correlation between female body size and the number of mature ovarian eggs. In other parasitoids, such as *Aphaereta minuta*, larger size has similarly been shown to benefit foraging in the field, to increase the number of eggs available, to increase egg size, to live longer and have a higher searching efficiency within patches (Visser, 1994). These findings show that influencing the insects' size can lead to positive benefits in terms of using them as biocontrol agents.

The cricket *Gryllus bimaculatus* is one of the species commercially reared by Monkfield Nutrition Ltd. They are sold in different life stages and sizes as feed for reptile pets. Although they are not used as biocontrol agents, their value lies in their use as feed or food (Alemu et al., 2017; Pambo et al., 2018). Enhancing the cricket's size might improve their commercial value by obtaining a higher mass animal from which create more end-products like cricket flour.

This thesis is not a study of honey bees, but it builds directly on results reported from manipulative studies on honey bee development (Kamakura 2011). Section 1.1 of this chapter introduces honey bees and how it is possible to obtain one of two different phenotypes from a given female egg, depending on the diet that the larva is subsequently fed. This leads to an investigation of the properties of royal jelly in section 1.2, which examines the importance of one of its proteins and the decision to use it in some of the experiments performed this thesis. Sections 1.3 and 1.4 explain the theory behind the techniques used in the subsequent chapters. Section 1.5 describes the species that were used to test the effects of those techniques.

## 1.1 DNA methylation and larval development

To test the role of epigenetics in the development of workers and queens, Kucharski and colleagues (2008) manipulated the production of the DNA cytosine-5-methyltransferase (DNMT) subfamily DNMT3, by silencing it with injections of *DNMT3* small interfering RNA (siRNA) into newly hatched larva. 72% of the treated subjects developed faster and emerged as queens with fully developed ovaries, the effects of royal jelly were thus mimicked. Further, RNAi treated queens and royal jelly grown queens were tested regarding growth and physio-metabolic-processes genes; it was found that similar genes were up-regulated in both cases. *DNMT3* knockdown thus seems to be able to replace royal jelly consumption, this may be an indication that at least one component of royal jelly affects activity of enzymes playing a role epigenetic gene regulation.

The expression of *DNMT* genes in adult bees is involved in social and physiological behaviours. Holman et al. (2016) analysed the effects of queen pheromones and linked it to a change in the workers methylome, with lower DNA methyltransferase 1 and 3 expression. This effect can lead to changes in the hive depending on the state of the queen, as described by Herb et al. (2013) as a reversible switch between epigenetic states. The role of *DNMT*s was also investigated regarding the role in the formation and consolidation of memory (Biergans et al., 2012, Biergans et al., 2015, Biergans et al., 2016), and olfactory ability and learning (Gong et al., 2016, Biergans et al., 2017). These studies show the importance of the DNA methylation mechanism in honey bees.

To measure the presence of epigenetic regulatory activity, Spannhoff et al. (2011) tested the effects of royal jelly on a specific cell line (K-ras-transformed NIH 3T3 cell reporter system) and gained expression of the proapoptotic *Fas* gene that is silenced in this cell line, thus confirming that royal jelly can influence the epigenetic state of some genes. To aid in the identification of possible factors that may influence epigenetic states, the group then fractioned royal jelly and analysed the different fractions; they found that the lower than 3 kDa weight fraction contained the component with epigenetic regulatory activity. Based on these results, it has been hypothesized that royal jelly's active component is not of proteinaceous origin, supported by the fact that once treated with proteinase K, royal jelly maintained its queen-inducing activity. The active component that they identified is the fatty acid 10-hydroxy-2-decenoic acid (10-HDA) (Figure 1.1), 10-HDA can compose between 2 and 6% of royal jelly and it possesses the ability to activate silenced genes on different cell lines (Spannhoff et al., 2011).

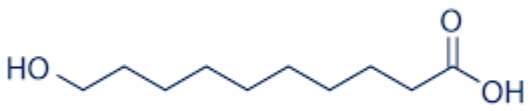



*Figure 1.1 10-HDA molecular structure*

The authors demonstrated that neither 10-HDA nor royal jelly are DNA methyltransferase inhibitors, as they were not able to reduce methylation *in vitro*. Finally, the group established the role of 10-HDA as a histone deacetylase inhibitor (HDACi): it blocks histone deacetylase (HDAC) activity, it has synergic functions with DNA demethylating agents, it is unable to inhibit DNA methylation and it has a similar structure to known HDACi compounds (Spannhoff et al., 2011).

10-HDA has been also linked to longevity in the nematode *Caenorhabditis elegans* (Honda et al., 2011, Wang et al., 2014, Honda et al., 2015), *D. melanogaster* (Gardner, 1948) and mice (Inoue et al., 2003); suggesting that the HDACi effects could interact with lifespan-control mechanisms conserved in diverse species (Kenyon, 2010).

To investigate the role of DNA methylation in defining honey bee caste phenotypes, Herb et al. ( 2013) compared the brain methylome of adult sister queen bees and worker bees showing no caste-specific differences, in direct contrast with the study of Lyko et al. ( 2010) where they found 560 differentially methylated expressed genes between queens and workers. However, Lyko et al. (2010) found differences between workers with age-dependent roles in the hive and suggested that the phenotypic shift could be

determined by a set of genes regulated by DNA methylation. The study also showed that it is possible to revert this phenotypic shift through DNA methylation, suggesting that bees belonging to different sub-castes might have specific methylation signatures.

## *1.2* Environmentally driven phenotypic plasticity in Honey bees (*Apis mellifera)*

To explain why royal jelly and its effects on insects are important, the life cycle of honey bees, and their ability to produce two phenotypically distinct female castes (queens and sterile workers) from one genotype, needs introduction. A queen bee performs one nuptial flight early in her lifetime during which she mates with up to 20 drones (males). After mating, the female stores sperm and subsequently has control over whether to lay fertilised or unfertilised eggs: fertilised eggs develop into females (workers or queens), while unfertilised eggs develop into male drones. Each female in the hive, excluding the queen bee, is either a sister or a half-sister, since the multiply-mater queen cannot select sperm from a particular male to fertilise her eggs. A fertilised (diploid) egg contains half of the maternal genome, with the other half coming from a drone. Unfertilized eggs are haploid, carrying half of the mother's genome only (Wang et al., 2016).

Honey bees have defined stages in their development (Winston, 1992) (Figure 1.2): egg, larva, and pupa, after which the adult emerges. The egg is laid into a brood cell by the queen and hatches after three days (Figure 1.2). Honey bees increase their size during the larval stage, which is the only time in which they are fed by specialised worker bees, called nurse bees which deposit the larval food at the bottom of the cell. Once the larva

is ready to pupate, the cell is operculated (closed) by nurse bees using a layer of beeswax. During the pupal stage, the developing bee undergoes metamorphosis into adulthood. Among adult females, there are two castes: queens that are specialised in reproduction and workers that are sterile.

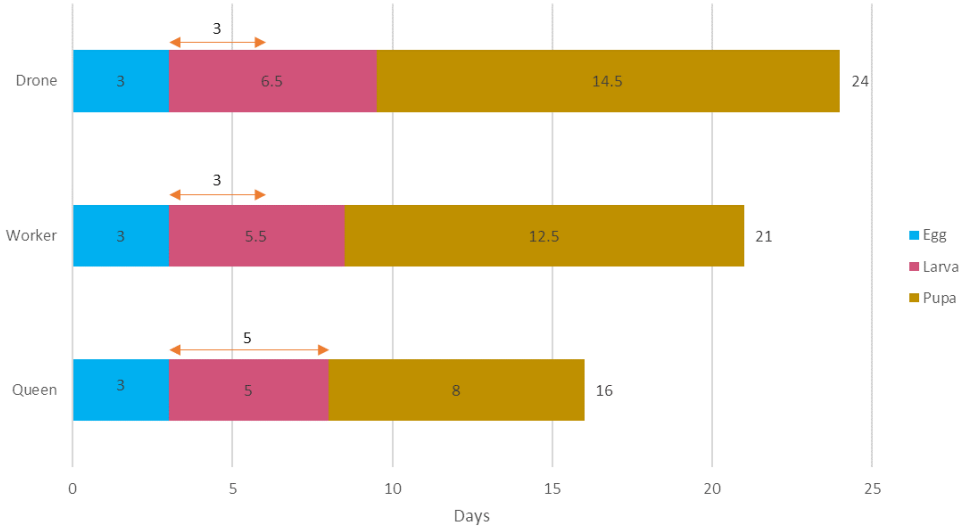



*Figure 1.2 Developmental times of honey bee castes from egg to adult. The red line above the larval phase indicates the number of days that the larva is fed royal jelly.*

The timing of development of queens and workers is different. For females (workers and queens), at least, developmental timing is influenced by the diet composition and quantity on the day of the instar. All female larvae are fed royal jelly from the first day after hatching from the egg until the third day. Royal jelly is secreted by young adult bees (burse bees) from specialised glands called hypopharyngeal glands. During the last days of the larval stage, the diet provided depends on whether the larva is destined to become a worker or a queen: if the lava is to develop into a queen, it is fed royal jelly for two more days. In case of a worker, for the remaining two and a half days the larva is fed exclusively pure with a mixture of royal jelly, pollen and honey, this food is called

beebread or worker jelly (Wang et al., 2016). Worker jelly (WJ) and royal jelly (RJ) compositions vary during the days that they are fed to the larvae, but in average RJ has a higher moisture content, a higher proportion of proteins, a higher proportion of 10-hydroxy-2-decenoic acid and finally a higher amount of fructose and glucose. Queen bees receive royal jelly not only for their entire larval stage, but also for the rest of their adult lives.

It is thus the diet of the female bee larvae that determines their development into a given adult caste. The diet fed to queens produces specific effects: their lifespan will last up to 20 times longer than their worker counterparts, their ovaries will develop, increasing their size, and will allow them to produce up to their bodyweight in eggs each day during the reproductive season. Queens also lack the specialised morphological and anatomical features of worker bees, such as the pollen baskets on their legs, hypopharyngeal glands and wax glands.

Royal jelly is mainly composed of water, ranging from 50% to 65%; proteins 11-18%; carbohydrates 10-15%; lipids 3-8%; salts 1.5% and small amounts of polyphenols and vitamins. The dry part is composed of 27–42% proteins, 22–31% carbohydrates, and 15–30% lipids (Karaali et al., 1988, Isidorov et al., 2012).

### 1.2.1 Epigenetics and the induction of queen-like phenotype in honey bees

While the trigger to the queen-like developmental modifications is diet, the underlying mechanism that establish this nutritionally driven phenotypic plasticity is mediated by epigenetics (Weiner and Toth, 2012, Welch and Lister, 2014). The definition of epigenetics, as put forward by Riggs et al. ( 1996) is “the study of mitotically and/or

meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence". This definition contrasts with an earlier definition by Waddington (1957): "how genotypes give rise to phenotypes during development". A more modern definition is given by Adrian Bird (2007) "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states", which is the definition adopted in this thesis as it is a mix of the previous ones and describes the present-day field of epigenetics.

There are three major mechanisms to generate epigenetic information in eukaryotic organisms: the methylation of DNA, the modification of histone proteins, and the activity of non-coding RNAs (Glastad et al., 2015, Glastad et al., 2019).

Animals possess two types of DNA methyltransferase (DNMT), the *de novo* methyltransferase performed by DNMT3 proteins, and the maintenance methyltransferase performed by DNMT1 proteins. *De novo* methylation happens on specific dinucleotides, the 5'-cytosine-phosphate-guanine-3' (CpG) pair, while DNMT1 restores the methylation on the newly synthetized strand after DNA replication (Lyko, 2018).

*Table 1.1 DNS methyltransferases functions.*

| DNA methyltransferase | Function |
|---|---|
| DNMT1 | Maintenance methylation of DNA |
| DNMT3 | *de novo* methylation of DNA |

The ability to methylate DNA in insects is variable; in Hymenoptera, for example, methylation differs consistently between groups: the primitively eusocial wasp *Polistes*

*dominula* shows the lack of DNMT3 and a reduced methylation ability compared to other Hymenopterans(Standage et al., 2016). Other Hymenoptera groups like bees, ants and sawflies show a higher methylation rate compared to wasps, with sawflies showing the highest percentage (Glastad et al., 2017). As mentioned before, the wasp *P. dominula* lost DNMT3, while honey bees possess three DNMTs, the same number as mammals, and finally fruit flies only possess one (DNMT2). Nevertheless, methylation is detectable in insects that do not possess DNMT3, this could be due to other DNMT enzymes obtaining new methylation functionality or due to methylation being transmitted between generations (Krauss and Reuter, 2011, Dunwell and Pfeifer, 2014).

Histone proteins form, together with DNA, a structure called the nucleosome (Talbert and Henikoff, 2010). These structures influence the expression of genes depending on their chemical modification at the N-termini of the histone tails. That is, histones can be modified after translation, replaced with a sequence variant, or displaced to reveal the genes that were wrapped around them (Glastad et al., 2019). The major histone proteins modifications are methylation or demethylation, acetylation or deacetylation, phosphorylation or dephosphorylation, which have been linked to a role in the regulation of social insect caste identity. For example, in a study on the ant *Camponotus floridanus* (Simola et al., 2013); the authors found a relationship between chromatin structure and the regulation of gene expression in the different castes.

The third type of epigenetic mechanism is mediated by non-coding RNAs (ncRNA) consisting of different classes, including long non-coding RNAs (lncRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and microRNAs (miRNAs). The first two types have been shown to be particularly effective in insects (Chambeyron

and Seitz, 2014), but have mostly been studied in *Drosophila*. lncRNA affects the organism through RNA interference, while piRNAs affect the acquisition and propagation of epigenetic changes through chromatin modifications.

*Table 1.2 Types of non-coding RNA that influence epigenetic modifications.*

| **Non-coding RNA** | |
|---|---|
| Long non-coding RNA | lncRNA |
| PIWI-interacting RNA | piRNA |
| Small interfering RNA | siRNA |
| Micro RNA | miRNA |

MicroRNA profiles were found to be different between queen bees and worker bees (Ashby et al., 2016), with the highest difference in profiles detected during the larva-to-pupa transition. The targets of these non-coding RNA were genes responsible of regulating cellular processes involved in neuronal differentiation, cell signalling and the formation of caste-specific structures. These genes are different from those controlled by DNA methylation. The authors proposed that the role of microRNAs could be to buffer the expression of genes to channel the individual to the right phenotype, meaning that they would be used to ensure the formation of the correct phenotype and to suppress the formation of alternatives (Ashby et al., 2016).

## 1.3 Royal jelly components and the development of honey bee larvae

### 1.3.1 Histone modifications and larval development

Dickman et al. (2013) analysed the post-translational modifications (PTMs) (methylation and acetylation) of honey bee histones and found 23 different modification states in 96-hour old larvae and in queen bee ovaries. They identified PTMs unique to queen bee

ovaries and suggested that the phenotypic plasticity could be mediated through the PTM of histones.

Huang et al. (2012) showed that royal jelly is rich in histone deacetylase inhibitors (HDACis) and theorised that this may be linked to the transformation of honey bee larvae into queens. They also tested the effect of DNA methyltransferase inhibitors (DMTis) and HDACi injections on larvae development. Both inhibitors were added to the diet of nurse workers producing royal jelly to affect gene expression in the glands responsible of royal jelly production. Their findings show that a diet including these inhibitors leads to a richer royal jelly produced by nurse workers, which enhances the growth of queen bee larvae. The resultant royal jelly is altered in its protein composition content, which allows queens to grow up to three times the normal size. It is possible that HDACis can regulate the composition of royal jelly by inhibiting enzymes such as histone acetyltransferases and histone deacetylases. The function of these two classes of enzymes is to change the conformation of chromatin and thus HDACis could influence this regulation and the ratio of the major royal jelly proteins (MRJPs) present in royal jelly (Huang et al., 2012).

Polsinelli and Yu (2018) showed a link between zinc, iron and potassium content in royal jelly and the queen bee differentiation, oxidative stress and longevity. All these activities are modulated by an inhibitory activity on histone deacetylase. The authors also exclude the possibility of a single queen-determining factor, in favour of a complex modulation of different factors, including metal ions (see above) and pathways that involve vitellogenin and juvenile hormone.

Wojciechowski et al. (2018) examined the relationship between the chromatin states in the honey bee larval stage and the caste that the larva will develop into. They created a map of chromatin structures 96h after hatching, representing the day when the caste fate of the larva is irreversible. They were able to discover that many of the different chromatin states are linked to caste-specific gene transcription. Some of the genes in the regions that differ between castes had not been identified before and could be related to caste identity.

### 1.3.2 Juvenile hormone, insulin/insulin-like signalling and target of rapamycin pathways and larval development

Another hypothesis concerning the queen-inducing effects of royal jelly is that its ingestion activates a series of phagostimulatory pathways leading to a higher intake of food and thus to a differential nutrition between workers and queens. It has been shown that a high intake of royal jelly, and more specifically the sugar content, activates the insulin/insulin-like growth factor (IIS) pathways (Koyama et al., 2013). The network activation leads to a rapid use of all the available nutrients and to an increased demand for more food. One of the hormones of the IIS pathway is the juvenile hormone (JH); Barchuk et al. (2007) theorised that the difference between workers and queens could be linked to the different expression of JH, such that workers' diet would not be able to raise the production of JH over the "queenliness" threshold. JH levels increase in the queen bee larva from 24h after hatching to the last instar, after which they fall (Rachinsky et al., 1990, Rembold et al., 1992). One of the roles of high JH levels is to inhibit the development of organs like the royal jelly producing glands, wax glands, and pollen collecting baskets which are not needed by queens (Maleszka, 2008). In queens,

the high JH titre also activates the expression of physio-metabolic genes and, together with the nutritional effect of royal jelly, they determine body growth. Low levels of JH induce a greater development of the brain in worker bees, required to perform tasks like gathering nectar or pollen. JH was shown to be able to induce queen bee development by topical and oral application (Dietz et al., 1979); it can also stimulate ovarian development in workers, thus enhancing body size, when used topically (Kamakura, 2011).

The link between juvenile hormone (JH), insulin/insulin-like signalling (IIS) and target of rapamycin (TOR) pathways was demonstrated by Mutti et al. (2011); they knocked out both IIS and TOR pathways using RNA interference in bee larvae fed on queen inducing diet and, as a result, JH levels decreased. Bees emerging from that experiment expressed the worker phenotype, while larvae that were subsequentially treated with topical application of JH developed with queen phenotypes. As a further confirmation on this association, Patel et al. (2007) showed that queen development is blocked if TOR is knocked out , probably due to the low levels of JH. A connection between JH and diet was drawn by Wheeler et al. (2006) who showed that there is a differential expression of IIS and TOR pathways between queen and worker larvae and consequently on the expression of JH.

These findings support the theory that the amount of sugar in royal jelly is the maker of the differentiation of queens, linking a high concentration of sugars in the diet to both a phagostimulatory effect and an activation of the JH pathway (Asencot and Lensky, 1976, Asencot and Lensky, 1985, Kaftanoglu et al., 2011).

### 1.3.3 Royalactin affects larval development through the EGFR pathway

In contrast to the earlier hypotheses, and in an attempt to show that the trigger to queen-like modifications in honey bees is a single component of royal jelly, Kamakura (2011) isolated the most abundant MRJPs and tested them individually. He fed the larvae a diet comprising a heat-treated royal jelly (to denature all the proteins) to which he added a single protein. The only instance in which he obtained queen bees was when using the 57 kDa glycoprotein major royal jelly protein 1 (MRJP1) that he renamed 'Royalactin'. He then added Royalactin to *Drosophila* (fruit fly) larval diet (20% of total diet) and obtained an increase in body size (body weight and body length) and fecundity. Further, fruit flies had an extended lifespan and shortened developmental time compared to those fed the control diet. Kamakura studied the pathway involved in the queen-like modifications caused by Royalactin by using epidermal growth factor receptor (EGFR) mutant *Drosophila* due to his previous studies in rat hepatocytes where he found that royalactin functions similarly to the Epidermal growth factor (Egf) (Kamakura, 2002; Kamakura et al., 2001). *Egfr* mutant fruit flies whose diet included Royal jelly showed a smaller body size and longer developmental time, compared to non-mutant flies that were fed the same diet, meaning that Royalactin acts on the epidermal growth factor pathway to produce those modifications. Kamakura showed that knockdown of *EGFR* in fruit flies using RNA interference (section 1.3) blocks the activation of the downstream pathway. If mutants of single EGFR downstream enzymes are fed Royalactin in their diet, they show either enlarged body size or faster development, meaning that Royalactin is influencing two separate pathways: one starting with the ribosomal protein S6 kinase (S6K) that affects body size, and the other

starting with the mitogen-activated protein kinase (MAPK) which affects developmental time. Kamakura (2011) also measured the titre of JH and observed that in *Drosophila* and in honey bees it was increased when the diet contained Royalactin.

Kamakura's findings were countermanded by Buttstedt et al. (2016) who repeated the experiments on honey bees and did not obtain any of Kamakura's results. They tested the ability of all MRJP to regulate queen development and showed that they are not essential for queen determination. The findings by Buttstedt et al. (2016) provide evidence against a single causal factor for queen differentiation and instead supports the hypothesis that it is a complex mixture of factors contained within the nutrition supplied in royal jelly and the amount and duration of this food that is ingested that determines the development of queens.

## 1.4 RNA interference

Chapter 5 describes the experiments performed to test the possibility of obtaining queen like modifications by knocking down the expression of a selection of genes using RNA interference. The rationale behind the use of this technique is that it is possible to reduce the expression of specific genes singularly to study the effects of each one of them. The targets that were chosen span between genes involved in insect development to EGFR, the gene that expresses the protein that is involved in the pathway which Royalactin acts upon, according to Kamakura (2011). This last gene knockdown was chosen to explore the possibility that reduction in the expressed protein would produce effects opposite to those that were obtained by Kamakura. Dabour et al. (2011) used RNA interference on one of the species used in this thesis, the

cricket *Gryllus bimaculatus*. By knocking down the expression of one single gene called FoxO, they were able to increase the insect's body size. This sets an important precedent in the modification of the insect's phenotype through the use of RNA interference and the study is of great importance in setting up the experiments performed in chapter 5, not only I try to replicate some of the results in that paper, but I expanded the list of target genes to include ones related to the studies done in the other chapter such as EGFR and Dnmt 1 and 3.

RNA interference (RNAi) is a process that involves a source of RNA to generate loss-of-function or reduced function on target genes by neutralising a target RNA containing a matching sequence (Fire et al., 1998, Kim and Rossi, 2008, Setten et al., 2019). The RNAi pathway can be exogenous or endogenous, depending on whether the DNA source comes from an extracellular source or if it is expressed by the cell itself. RNAi can be triggered by three classes of small RNA molecules, as summarized in Table 1.3.

*Table 1.3.1 RNA interference types.*

| **RNAi type** | **Function** |
|---|---|
| MicroRNA (miRNA) | Genome encoded; regulate a multitude of cellular processes |
| PIWI-interacting RNA (piRNA) | Silence transcripts derived from selfish genomic elements (such as transposons) |
| Small interfering RNA (siRNA) | Defend the organism against invading viruses |

For the purpose of this thesis, RNAi will henceforth refer to the siRNA interference approach (Figure 1.3). The siRNA interference response is triggered when a double stranded RNA (dsRNA) is recognised in the cytoplasm, the dsRNA is cut by an RNase,

called Dicer, into 20 to 25bp siRNA. Then a series of proteins couple together to create the RNA-induced silencing complex (RISC) which links to the siRNAs. The RISC-siRNA complex loses the passenger strand of the siRNA and then it links to the complementary region of the target mRNA and degrades it (Bernstein et al., 2001, Rana, 2007, Cooper et al., 2019).

Most of RNAi experiments on insects focus on one of three areas: embryonic development, post-embryonic development, and resistance against xenobiotic chemicals. Experiments belonging to the last group have a great importance for pest control as these studies can shed light on the mechanisms of action of insecticides, or even discover new ways to target pests (Gatehouse et al., 2011, Joga et al., 2016, Niu et al., 2018).

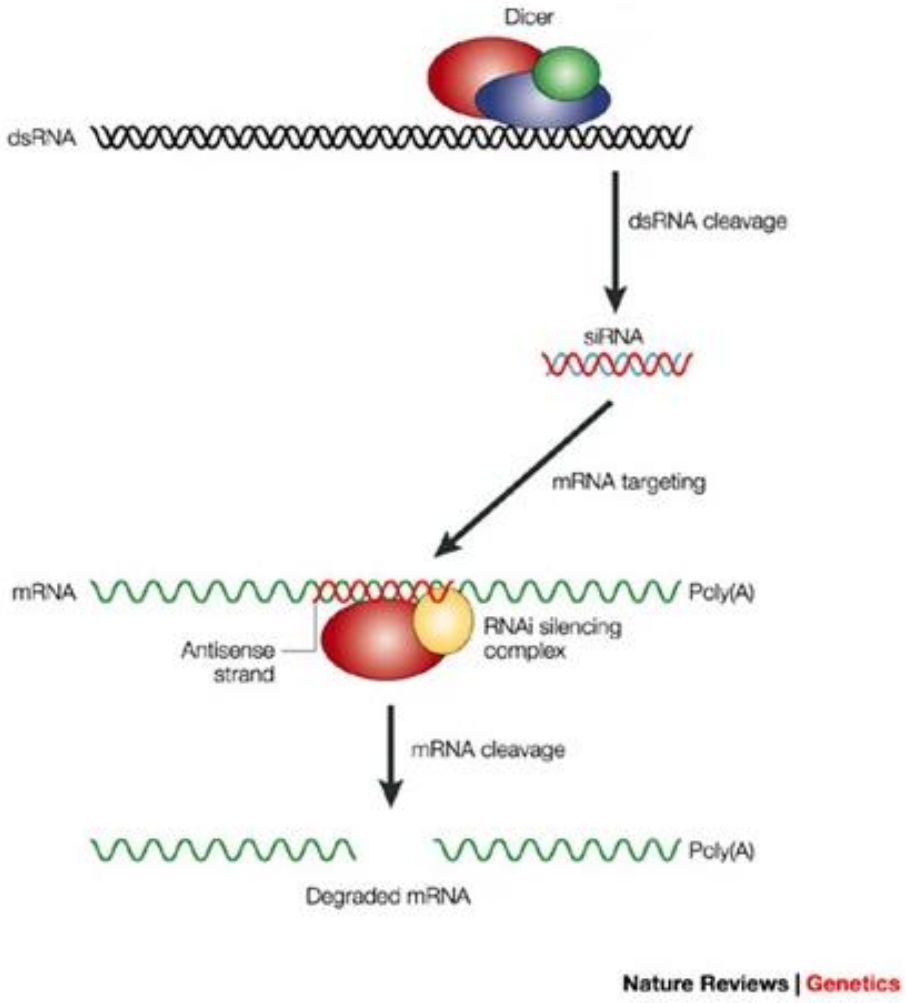



*Figure 1.3 siRNA interfering process. Long double-stranded RNA (dsRNA) is processed by the Dicer complex to form siRNAs. The antisense strand of the siRNA is used by an RNA interference (RNAi) silencing complex (RISC) to guide mRNA cleavage, so promoting mRNA degradation. Image taken from McManus & Sharp (2002)*

One of the problems with RNAi treatment in insects is the great variation in efficiency. A lack of response may not be linked to the deficiency of the RNAi core machinery and is more likely to be due to (a) the inability of the dsRNA to penetrate cells and tissues, (b) the lower expression of genes involved in the machinery, (c) the transcription rates

of the target gene, or (d) to the degradation mechanisms unique to insect species (Belles, 2010, Garbutt et al., 2013, Wang et al., 2016).

## 1.5 DNA methylation inhibitors to induce phenotypic variations.

Chapter 4 reports my findings on the use of DNA methylation inhibitors to modify the phenotype in insects. The hypothesis investigated is that it may be possible to obtain similar effects to those obtainable by feeding Royalactin to honey bee larvae by modifying the methylation of the insects' DNA. The aim is to find an alternative way to obtain the queen-like effects that Kamakura (2011) reported after adding Royalactin to the insect diet: this is done by inhibiting DNA methylation, similarly to the work of Kucharski et al. (2008). The queen-like effects obtained using DNA methylation inhibitors are comparable to those of royal jelly, as shown by Spannhoff et al. (2011). Not having to depend on the use of royal jelly, or on the production of its most abundant protein Royalactin to obtain these effects could prove cheaper and faster, since the collection of royal jelly is a long and expensive process and the expression of recombinant Royalactin will always be longer than using pre-manufactured chemicals. The compounds used are 5-azacytidine and RG108: both inhibit DNA methylation, but with two different modes of action due to the conformation of the molecules. These two compounds decrease the action of the enzyme DNA methyltransferase, which in turn reduces the methylation of the DNA allowing the expression of different sets of genes.

### 1.5.1 5-azacytidine

Compounds of the family of the azacytidines were first developed as cytostatic agents and then characterised as DNA methyltransferase inhibitors (DNMTi) in 1980 (Schapira and Arrowsmith, 2016). 5-azacytidine is mostly used as a cancer chemotherapeutic agent, due to its capacity to modulate some of the tumour's phenotypes (Jones, 1985). 5- azacytidine is a nucleoside analogue with DNA methyltransferase inhibitor properties, as it acts as a cytosine substitute during DNA duplication (Figure 1.4). DNA methyltransferases (DNMTs) normally attach to a cytosine and transfer a methyl group from a donor to the base; the enzyme then releases the substrate and is ready for a new reaction. The chemical structure of 5-azacytidine can accept the methyl group but prevents the enzymatic action of DNMTs to release from the substrate; the trapped enzyme becomes degraded, causing the depletion of DMNTs in the cell, which in turn leads to demethylation and expression of certain genes in cells where they would be normally silenced (Stresemann et al., 2006, Stresemann and Lyko, 2008). The incorporation of 5-azacytidine into the DNA is random and can affect different locations of the genome, depending on where and when it is incorporated instead of cytosine (Yang et al., 2010).

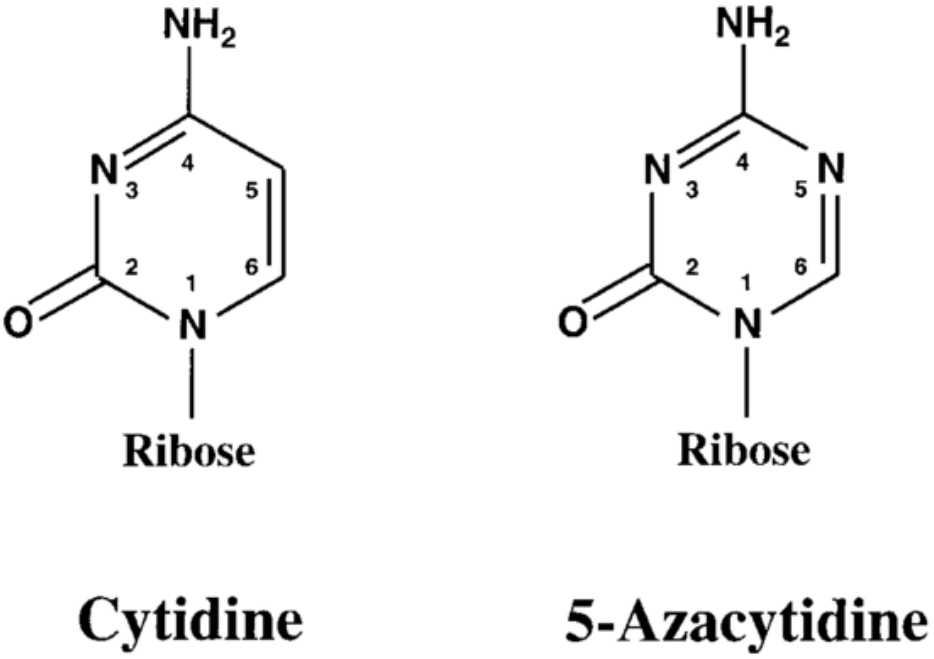



*Figure 1.4 Chemical structure of the nucleoside cytidine and of its inhibitor 5-azacytidine*

### 1.5.2 RG108

RG108 is a synthesised human DNMT1 inhibitor (Figure 1.5), it was designed to strongly interact with its active site, interfering with enzyme activity, but without depleting it (Brueckner et al., 2005). Although it was designed based on a human enzyme, the fact that the DNMT family is conserved makes it possible to use RG108 on insects as well. In honey bees, RG108 has a lifespan increase effect without influencing the caloric intake or the juvenile hormone titre (Cardoso-Junior et al., 2018). This is a potential beneficial side effect that could interest the industrial partner, as the produced insects could have longer shelf-life.

The two chemicals have different toxicity and demethylation effects: 5-azacytidine is known for its cytotoxicity, genotoxicity, and apoptosis induction, but it also shows a

much stronger demethylation effect compared to the non-toxic RG108 (Stresemann et al., 2006).

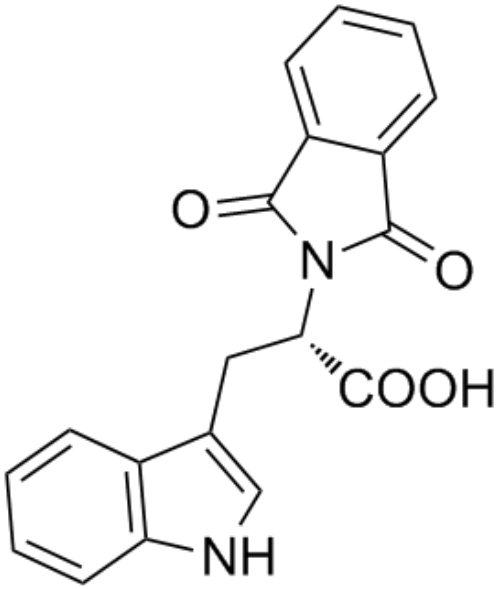



*Figure 1.5 Chemical structure of the human DNA methyltransferase enzyme RG108*

## 1.6 Non-model species

Two insect species were used in experiments: *Goniozus legneri* and *Gryllus bimaculatus*.

### 1.6.1 *Goniozus legneri*

The parasitoid wasp *Goniozus legneri* Gordh belongs to the Aculeate hymenopteran family Bethylidae. Bethylids are ectoparasitoids of lepidopteran and coleopteran larvae and are used as biological control agents against several agricultural pests in these insect orders (Gifford, 1965, Gordh and Evans, 1976). As with other hymenopterans, *G. legneri* is a holometabolous species, meaning that it goes through a complete metamorphosis with four major life-history stages: egg, pupa, larva, and adult.

As aculeates, bethylids are more closely related (phylogenetically) to the highly social honey bees than are the majority of parasitoid hymenopterans (Peters et al. 2017). Many bethylids, including *G. legneri*, exhibit sub-social behaviour (maternal care via post-ovipositional brood guarding), a relatively rare trait among parasitic wasps (Hardy and Blackburn, 1991). Kamakura used *Drosophila* to attempt to replicate the effects of Royalactin in a different insect species (Kamakura, 2011), but *Drosophila* are phylogenetically distant from *Apis*; using *G. legneri* provides an evaluation in a more closely related species within the same insect order as *Apis*. Further, *G. legneri* is simple to maintain under laboratory conditions, has a short generation time and aspects of its developmental biology and behaviour are well understood (, Hardy et al., 2000, Goubalt et al., 2006, Du et al., 2021).

#### *1.6.1.1* Life cycle and biology of *Goniozus legneri*

Once an adult female of *G. legneri* finds a prey, it paralyses it by stinging it and around a day later it lays eggs on it. The eggs hatch after approximately a day into larvae, which attach their mouth parts to the integument of the prey and feed on it ecto-parasitically. After feeding on the host, the larvae detach and spin a cocoon and then metamorphose into pupae. The length of their development is related to the surrounding temperature (Butler and Schmidt, 1985), with the duration of the egg-to-prepupal phase lasting 12.8 days at 17°C and 3.1 days at 35°C and the pupal-to-adult phase lasting 35 days at 17°C and 5.0 days at 35°C. Once emerged from the cocoons, the brood disperses, usually after mating: there are typically one or a few males in each brood and these mate with their more numerous sisters (Hardy et al., 2000).

### 1.6.2 *Gryllus bimaculatus*

*Gryllus bimaculatus* De Geer (order Orthoptera, family Gryllidae) was chosen as a non-model species because it is one of the species that is commercially produced by Monkfield Nutrition Ltd., the insect-rearing company involved in this research programme.

A study has been carried on studying the effect of royal jelly on *G. bimaculatus*, showing body enlarging effects (Miyashita et al., 2016), but no specific studies on the role of Royalactin have been carried out. It is also worth noting that RNA interference experiments have been carried on *G. bimaculatus*, showing that the species reacts positively to the treatments (Dabour et al., 2011, Hamada et al., 2015).

#### *1.6.2.1* Life cycle and biology of *Gryllus bimaculatus*

In contrast to honey bees and *G. legneri*, which are holometabolous, *G. bimaculatus* has a hemimetabolous life cycle (lacking a pupal stage). The egg hatches into a nymph that, through several moults, develops into an adult. Aside from their smaller size, nymphs are broadly similar in appearance to adults but lack functional reproductive organs and wings. It takes 12 days for a fertilised egg to hatch into a first instar *G. bimaculatus*, then the larva goes through eight more moults and reaches adulthood seven weeks after the egg was laid. Male and females can only be sexed when they reach adulthood once they develop their reproductive organs. They can be easily distinguished due to the difference in size (females are larger) and the presence of the ovipositor, which is only present in females.

## 1.7 Summary of thesis structure

The general question that is fundamental to this project is if it is possible to influence the phenotype of adult insects to obtain an increase in performance-related parameters such as body size, weight, and lifespan.

Specific aims are to determine if by adding Royalactin to the insects' feed, by using DNA methyltransferase inhibitors, or by using RNA interference on development genes, insect development (in particular body weight) can be increased.

In summary, the structure of the thesis is as follows:

Chapter 2 lists all materials and methods that were used performing the experiments. In each empirical chapter there is a section that gives more detail on methods exclusive to that chapter.

Chapter 3 examines the procedures to express Royalactin using *E. coli* cell lines and its purification. The aim is to assess whether it is possible to reproduce the expression of recombinant Royalactin as reported by Kamakura (Kamakura, 2011) and, if successful, add the purified protein to insects' diet to try to promote the occurrence of queen-like phenotypes.

Chapter 4 evaluates the effects of inhibiting DNA methylation on *G. legneri* and *G. bimaculatus* using the chemicals 5-azacytidine and Rg108. The aim is to obtain queen-like phenotypes by decreasing the methylation, as shown in the paper (Kucharski et al., 2008).

Chapter 5 investigates the production of dsRNA probes to knock out specific genes in *G. legneri* and *G. bimaculatus* during their development. The aim is to assess whether the knockdown of these genes is inducing new phenotypes in these two organisms, and to understand the molecular pathways of the queen-like effects by using genes.

Chapter 6 provides a general discussion and conclusions arising from the experiments reported in this thesis.

# Chapter 2 Materials and methods

This chapter lists the materials and methods used in the experiments described in this thesis. Section 2.1 lists all the materials used as well as their manufacturer, separated for each technique. Sections 2.2 lists the techniques used in chronological order.

## 2.1 Materials

### 2.1.1 *Escherichia coli* cell lines

Four engineered *E. coli* cells lines were used to express the protein Royalactin. These strains are listed in Table 2.1.

*Table 2.1 E. coli cell lines.*

| Name | Manufacturer |
|---|---|
| BL21 (DE3) Competent Cells | NEB |
| Origami 2 (DE3) *pLysS* Competent Cells | Novagen |
| Shuffle T7 Express *lys Y* Competent Cells | NEB |
| Lemo21 (DE3) Competent Cells | NEB |

### 2.1.2 Western blot antibodies

Two sets of antibodies were used for western blots, the first pair was the His-tag unconjugated rabbit polyclonal antibody and the goat to rabbit HRP-conjugated polyclonal antibody. The second pair was the mouse monoclonal antibody to 6XHis-tag and the Goat polyclonal antibody to mouse IgG (HRP) (Table 2.2).

*Table 2.2 Western blot primary and secondary antibodies.*

| **Primary antibodies** | **Manufacturer** | **Dilution for WB** |
|---|---|---|
| His-tag unconjugated rabbit polyclonal antibody, LS-C137154 | LSBio | 1:2000 |
| Mouse monoclonal antibody to 6XHis-tag, Ab 18184 | ABCAM | 1:2000 |
| **Secondary antibodies** | **Manufacturer** | **Dilution for WB** |
| Goat to rabbit HRP-conjugated polyclonal antibody, LS-C60885 | LSBio | 1:20000 |
| Goat polyclonal antibody to mouse IgG (HRP), Ab 205719 | ABCAM | 1:20000 |

### 2.1.3 Chemicals

Table 2.3 lists in alphabetical order all the chemicals and their manufacturers that were used in the experiments described in this thesis.

*Table 2.3 Chemicals.*

| **Chemical** | **Manufacturer** |
|---|---|
| 5-azacytidine, Hybri-Max™, γ-irradiated, lyophilized powder, BioXtra | Sigma-Aldrich |
| Acetic acid, glacial | Scientific Laboratory Supplies |
| Acrylamide 30% | Flowgen Biosciences |
| Agar | Calbiochem |
| Agarose | Sigma |
| Ammonium persulphate (APS) | Sigma |
| BCA protein assay Solution A | Fisher Scientific |
| BCA protein assay Solution B | Fisher Scientific |
| Bovine serum albumin (BSA) | VWR |
| Calcium chloride (CaCl) | Sigma |
| Chloroform | Fluka Analytical |
| Coomassie Blue G-250 | Fisher Scientific |
| Dimethyl sulfoxide (DMSO) | Sigma |
| Dithiothreitol (DTT) | Sigma |
| Ethanol (EtOH) | Fisher Scientific |
| Ethidium bromide | Fisher Scientific |
| Ethylenediaminetetraacetic acid (EDTA) | Fisher Scientific |
| Glycerol | Fisher Scientific |
| Glycine | Sigma |
| Imidazole | Fluka Analytical |

| | |
|---|---|
| InstantBlue | Expedeon |
| Isoamyl alcohol | Sigma |

*Table 2.4 Chemicals (cont.).*

| | |
|---|---|
| Isopropyl-β-D-thiogalactopyranoside (IPTG) | Fisher Scientific |
| Methanol (MeOH) | Fisher Scientific |
| PBS tablets | Sigma |
| Phenol, acid | Ambion |
| Phenyl-methyl-sulfonyl fluoride (PMSF) | Sigma |
| Potassium chloride (KCl) | Fisher Scientific |
| Resolving gel buffer | Bio Rad |
| RG108 | Abcam |
| RNaseZap | Ambion |
| SimplyBlue SafeStain | Fisher Scientific |
| Sodium azide (NaN3) | Fisher Scientific |
| Sodium chloride (NaCl) | Fisher Scientific |
| Sodium dihydrogen phosphate | Fisher Scientific |
| Sodium dodecylsulphate (SDS) | Sigma |
| Stacking gel buffer | Bio Rad |
| Sucrose | Sigma |
| Tetramethylethylenediamine (TEMED) | Fisher Scientific |
| TGS 10X (Tris-Glycine-SDS) | Scientific Laboratory Supplies |
| Trisaminomethane (Tris)-HCl | Fisher Scientific |
| Triton X-100 | Sigma |
| Tryptone | Oxoid |
| Tween 20 | Sigma Aldrich |
| Urea | Fluka Analytical |
| X-gal | Promega |
| Yeast extract | Oxoid |

### 2.1.4 Buffers and mediums

Table 2.5 lists all the buffers and their composition, used in the methods sections (section 2.2).

*Table 2.5 Buffers and mediums.*

| Buffer or medium | Ingredients |
|---|---|
| 2× Laemmli buffer | 4% w/v 10% SDS<br>20% v/v Glycerol<br>120mM Tris-HCl pH 6.8 |
| Inclusion bodies solution 1 | 0.25M Tris-HCl pH 8.0<br>0.5M NaCl<br>5M EDTA |
| Injection buffer | 0.1 mM sodium phosphate<br>5mM KCl<br>pH 6.8 |
| Lysogeny broth (LB) medium 1L | 10g w/v tryptone<br>5g w/v yeast extract<br>5g w/v sodium chloride |
| LB agar | LB medium<br>1.5% w/v agar |
| Lysis buffer | 50mM Tris-HCl pH 8.0<br>100mM NaCl<br>5mM EDTA<br>0.1% w/v sodium azide<br>0.5% v/v Triton X-100<br>0.1mM PMSF<br>1mM DTT added freshly |
| Nickel affinity cartridge binding buffer | 20mM sodium phosphate<br>500mM NaCl<br>10mM imidazole<br>pH 7.5 |
| Nickel affinity cartridge elution buffer | 20mM disodium phosphate<br>500mM NaCl<br>500mM imidazole<br>pH 7.5 |
| Nickel affinity cartridge elution buffer, denaturing conditions | 50mM sodium phosphate<br>300mM NaCl<br>250mM imidazole<br>8M urea<br>pH 8.0 |
| Nickel affinity cartridge equilibration and binding buffer, denaturing conditions | 50mM sodium phosphate<br>300mM NaCl<br>10mM imidazole<br>pH 8.0 |
| Nickel affinity cartridge wash buffer, denaturing conditions | 50mM sodium phosphate<br>300mM NaCl<br>20mM imidazole<br>8M urea<br>pH 8.0 |

*Table 2.6 Buffers and mediums (cont.).*

| | |
|---|---|
| Nickel magnetic beads binding/washing buffer | 50 mM sodium phosphate<br>300 mM NaCl<br>20 mM imidazole |
| Nickel magnetic beads elution buffer | 50 mM sodium phosphate<br>300 mM NaCl<br>500 mM imidazole |
| TAE (Tris base, acetic acid, EDTA) buffer | 40mM Tris<br>20mM glacial acetic acid<br>1mM EDTA |
| TBS (tris buffered saline | 50 mM Tris-Cl, pH 7.5<br>150 mM NaCl |
| TBS-T (tris-buffered saline, Tween 20) | 1% v/v TBS 10×<br>0.1% v/v Tween 20 |
| TBS-T BSA | 3% w/v BSA<br>97% v/vTBS-T |
| TGS (Tris-Glycine-SDS) buffer | 25 mM Tris-HCl<br>250 mM glycine<br>0.1% v/v SDS |
| Western blot transfer buffer | 10% v/v TGS buffer<br>20% v/v methanol |

### 2.1.5 SDS-PAGE protein markers

Table 2.7 lists the protein markers used to determine the size of the proteins ran in the SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels (section 2.2.12).

*Table 2.7 SDS-PAGE protein markers*

| Marker | Manufacturer |
|---|---|
| Precision Plus protein dual colour standards | Bio Rad |
| Unstained precision plus protein standard | Bio Rad |

### 2.1.6 DNA electrophoresis marker

Table 2.8 lists the DNA marker used to determine the size of the DNA ran in the electrophoresis gels (section 2.2.16).

*Table 2.8 DNA electrophoresis marker.*

| Marker | Manufacturer |
|---|---|
| 2-Log DNA Ladder (0.1–10.0 kb) | NEB |

### 2.1.7 DNA electrophoresis loading buffers.

Table 2.9 lists the loading buffers used to dye and increase the density of the samples to help visualising and running them in an electrophoresis (sections 2.2.12 and 2.2.16).

*Table 2.9 SDS-PAGE loading buffers.*

| Loading buffer | Manufacturer |
|---|---|
| 6× loading buffer | New England Biolabs |
| 10× loading buffer | New England Biolabs |

### 2.1.8 Western blot marker

Table 2.10 lists the marker used to determine the molecular weight of the proteins ran in a western blot (section 2.2.19).

*Table 2.10 Western blot marker.*

| Marker | Manufacturer |
|---|---|
| Amersham ECL Dualvue Western Blotting marker | GE Healthcare |

### 2.1.9 Western blot materials

Table 2.11 lists the consumables and their manufacturers needed to set up a western blot reaction (section 2.2.19).

*Table 2.11 Western blot materials.*

| Name | Manufacturer |
|---|---|
| Amersham Hybond® ECL™ nitrocellulose membrane | GE Healthcare |
| Clarity Western Peroxide Reagent | Bio-Rad |
| Clarity Western Luminol/Enhancer Reagent | Bio-Rad |
| S-protein HRP-conjugated (1:20000) | Abcam |

### 2.1.10 Antibiotics

Table 2.12 lists the antibiotics used to selectively grow the *E. coli* lines described in section 2.1.1 (different cell lines require different antibiotics).

*Table 2.12 Antibiotics.*

| Antibiotic | Manufacturer | Working concentration |
|---|---|---|
| Ampicillin | Sigma | 100 µg/mL |
| Kanamycin | Sigma | 50 µg/mL |
| Carbenicillin | Tocris Biosciences | 100 µg/mL |
| Chloramphenicol | Sigma | 25 µg/mL |

### 2.1.11 Vectors

Table 2.13 lists the vectors used to transform *E. coli* cell lines (pET28b, Figure 2.1) and to cloning PCR products for sequencing (pCR4-TOPO TA, Figure 2.2).

*Table 2.13 Vectors.*

| Vector | Manufacturer | Length | Resistance | Characteristics |
|---|---|---|---|---|
| pET28b | Novagen | 5368 bp | Kanamycin | N-terminal and a C-terminal His-Tag sequence, multiple cloning site |
| pCR4-TOPO TA | Invitrogen | 3956 bp | Ampicillin and kanamycin | M13 forward and reverse sequences, and T7 promoter and terminator |

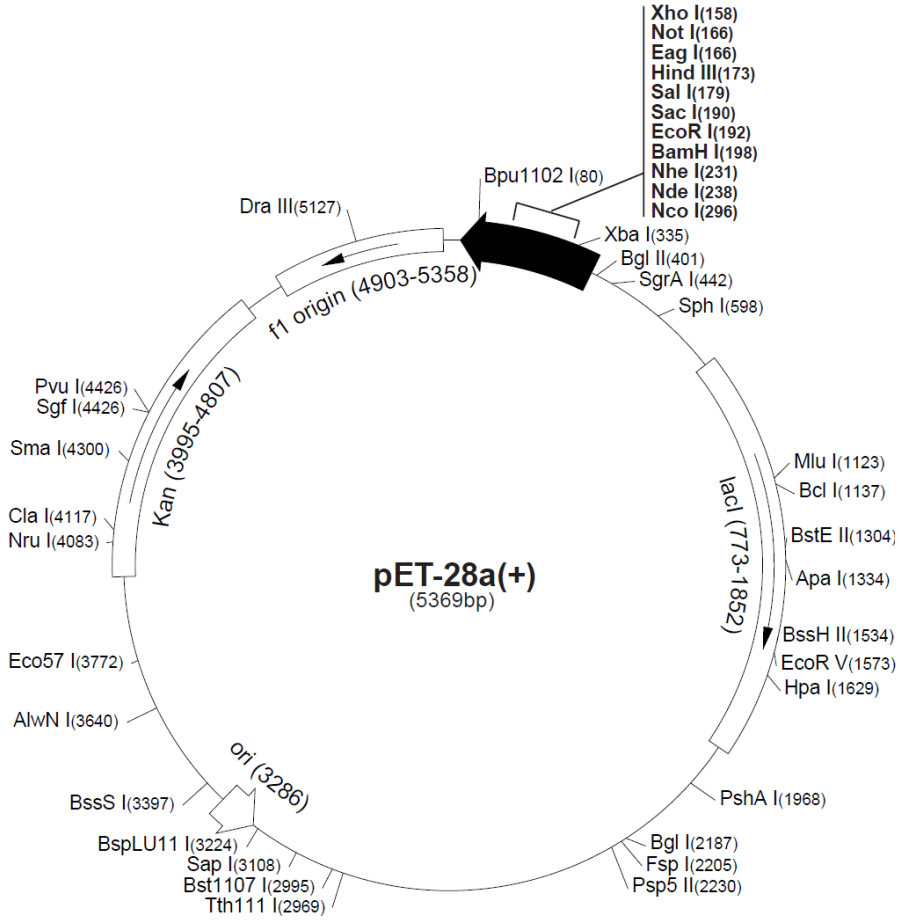



*Figure 2.1 Illustration of pET-28a vector, to obtain pET-28b, subtract 1bp from each site beyond Bam HI at 198bp. Figure adapted from Novagen.*

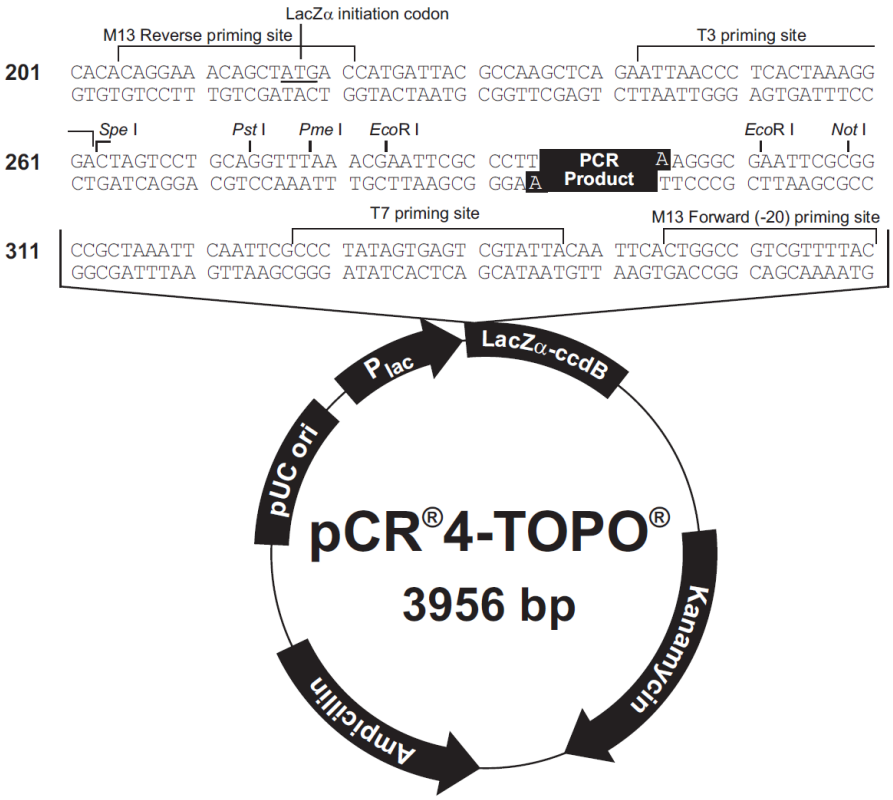



*Figure 2.2 Illustration of pCR4-TOPO TA vector and the insert site. Figure adapted from Invitrogen.*

### 2.1.12 PCR materials

All PCR reactions were performed using HotStarTaq DNA Polymerase (Qiagen). This polymerase is a Hot-start (HS) polymerase that needs 15 minutes activation at 95°C, allowing reactions to be set up at room temperature with no polymerase activity at room temperature. Table 2.14 lists the materials needed to perform a PCR reaction.

*Table 2.14 PCR materials.*

| **Component** | **Manufacturer** |
|---|---|
| HotStarTaq DNA Polymerase | Qiagen |
| 10× PCR buffer | Qiagen |
| dNTPs | Promega |
| PCR grade water | Ambion |
| Primers | Eurofins Genomics |

The 10× PCR buffer provides a final concentration of 1.5mM magnesium chloride ($MgCl_2$). The reaction followed the manufacturer's specification:

*Table 2.15 PCR reaction component final concentration*

| **Component** | **Final concentration** |
|---|---|
| HotStarTaq DNA Polymerase | 2.5 units/reaction |
| 10× PCR buffer | 1× |
| dNTPs | 200 µM each |
| Primers | 4 µM each |

### 2.1.13 Oligonucleotides

This section describes all the primers used in this thesis. All primers were provided by Eurofins Genomics (Ebersberg, Germany) and upon arrival were suspended to create a 100 µM stock solutions with PCR grade water. Stock solutions were then kept in the freezer.

#### 2.1.13.1 Insert-integrity primers.

All primers in Table 2.16 were designed by Dr Reinhard Stöger. These primers were used to sequence the vector containing the DNA target sequence, to make sure that the cloning was performed correctly.

*Table 2.16 Insert- integrity primers.*

| Name | Forward primer 5’ - 3’ |
|---|---|
| pET28-1 | AAGCGCTCATGAGCCCG |
| MRJP-F1 | CCGACATTGACCAATGGC |
| MRJP-F2 | GCCACTACAGGAAAGGG |
| MRJP-F3 | GGAACAATTCAGAACATCCG |
| MRJP-F4 | AAGCTCTTCCACACGTGCC |

### 2.1.13.2 Degenerate PCR primers

All primers in Table 2.17 were designed by me, except *DNMT*1 that was designed by Dr Reinhard Stöger. Degenerate primers are primers that are designed as to be redundant in their sequence to cover all possible nucleotide combinations for a given sequence which is only partially known. These degenerate primers were used to elongate genes whose sequences were unknown in the target species that I used.

*Table 2.17 Degenerate PCR primers.*

| Name | Forward primer 5’ - 3’ | Reverse primer 5’ - 3’ |
|---|---|---|
| FoxO | CSCGNCGSAAYGCNTGGGGWAAYC | TTCTCRWAYYKRSDMGTCTCCATBG |
| EGFR | TACACSAACTGYACBTAYGTBGA | GCGCARAAVAGRTGRCARCAYTC |
| DNMT 1 | YGGWGGDHVBGGWVDWMRHAAR | HHDNGRRTYHGMNGTYTCDCCNARHAYDG |
| DNMT 3 | TGGTGGCCMKSRRTKATHRTYGAT | CAYANDGAWSCYWYRAARAAYGGATG |
| Myoglianin | GRAMCRTRGGHCTBAAYTGCGAYGA | CANYKMTCSACNAYCATRCCYGGYA |

Table 2.18 lists the amended degenerated primers to reduce degeneracy. All primers were designed by me.

*Table 2.18 Amended degenerated primers.*

| Name | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|
| *DNMT* 3 | TGGTGGCCMGCRATGATTATYGAT | CAYAAAGATCCYWCRAAGAAYGGATG |
| *Myoglianin* | GRAACGTRGGACTYAAYTGCGAYGA | CATYTCTCGACGACCATGTTGTG |

### 2.1.13.3 Species-specific and gene-specific PCR primers

This section lists the species-specific primers for both *Goniozus legneri* (Table 2.19) and *Gryllus bimaculatus* (Table 2.20). It also lists the primers for the negative control DsRed red fluorescent protein (Table 2.21).

#### *2.1.13.3.1 Goniozus legneri*

*Table 2.19 G. legneri species specific primers.*

| Name | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|
| Wasp-*FoxO* | CGCGGCGGAATGCGTGG | TTCTCGAATCGGGGCGTCTC |
| Wasp-*EGFR* | TACACGAACTGTACCTACGTGG | GCGCAGAAGAGGTGGCAG |
| Wasp-*DNMT*1-1 | CGAAGAAGATCCTTCTGCAGAGGG | CTGTGAATCTAGACAACCCTTGTGG |
| Wasp-*DNMT*1-2 | CGAAGAAGATCCTTCTGCAGAGGG | CTGTGAATCTAGACAACCCTTGTGG |
| Wasp-*DNMT*3-1 | AGATATTATTCAAAGATCCGTGGG | TAGCCAGACTTAAGTCATTACAGGG |
| Wasp-*DNMT*3-2 | GTCGCCAAAGTCATGTATATGTGGG | GGAGAAGAATTCCGCGTCGGCAAC |
| Wasp-*Myoglianin* | GGAACGTGGGCCTTAACTGC | CATTTATCGACTATCATGCCTGG |

2.1.13.3.2 Gryllus bimaculatus

*Table 2.20 G. bimaculatus species specific primers.*

| Name | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|
| Cricket-*FoxO* | CGCGGCGGAACGCGT | TTCTCGTACCTGGGCGTCTCCATTG |
| Cricket-*EGFR* | TACACGAACTGTACGTATGTGGA | GCGCAGAACAGGTGGCAGCATTC |
| Cricket-*DNMT*1-1 | CGAAGAAGATCCTTCTGCAGAGGG | CTGTGAATCTAGACAACCCTTGTGG |
| Cricket-*DNMT*1-1 | CGAAGAAGATCCTTCTGCAGAGGG | CTGTGAATCTAGACAACCCTTGTGG |
| Cricket-*DNMT*3-1 | AGATATTATTCAAAGATCCGTGGG | TAGCCAGACTTAAGTCATTACAGGG |
| Cricket-*DNMT*3-2 | GTCGCCAAAGTCATGTATATGTGGG | GGAGAAGAATTCCGCGTCGGCAAC |

2.1.13.3.3 *Discosoma* DsRed negative control primers

*Table 2.21 Discosoma red fluorescent protein primers.*

| Name | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|
| *DsRed* | CGACTACAAGAAGCTGTCCTTC | TTGGAGTCCACGTAGTAGTAGCC |

## 2.2 Methods

### 2.2.1 Preparation of LB agar Petri dishes

LB agar selection plates were prepared by heating a LB agar bottle (section 2.1.4) in a microwave until all the agar was dissolved. The agar bottle's temperature was reduced by placing it into a water bath set at 50°C and, working in sterile conditions, the required amount was transferred to a smaller container where the medium was mixed to the appropriate antibiotics. The plates were left to set for 30 minutes and then put upside down in the oven to dry for around an hour. The plates were used or stored at 4°C.

### 2.2.2 Streaking of *E. coli* cells on a LB agar Petri dish

A selection plate containing the appropriate antibiotics was warmed up in the oven to the bacterial cell line's growth temperature and streaked using a single cell under sterile conditions. The plate was then put in the oven and grown overnight at the appropriate temperature. Plates containing *E. coli* cells were taped with Parafilm and then stored at 4°C.

### 2.2.3 Production of *E. coli* calcium chloride competent cells

A single colony or 1 mL of an overnight culture were used to inoculate 100 mL of LB in a 500 mL flask which was incubated shaking at the appropriate temperature for 3 hours. After that, the cells were put on ice for 10 minutes and kept cold from now on. Cells were collected by centrifugation for 15 minutes at 6000 g at 4°C, then the supernatant was discarded, and cells were gently resuspended in 10 mL cold 0.1M $CaCl_2$. Cells were incubated on ice for 20 minutes and then centrifuged for 15 minutes at 6000 g at 4°C and the supernatant was discarded. The cell pellet was then gently resuspended with 2 mL of cold 0.1 M $CaCl_2$ and 15% v/v Glycerol solution and stored at -80°C or immediately used for transformation.

### 2.2.4 Transformation of *E. coli* competent cells

1 µl of pET28b plasmid containing 1 pg–100 ng of Royalactin's DNA was added to 100 µL of competent cells, the tube was gently flicked 4 to 5 times and incubated for 30 minutes on ice. The cells were heat shocked for the appropriate time (Table 2.22) in a 42°C water bath and then cooled on ice for 5 minutes. 700 µL of room temperature SOC or LB medium were added to the tube, which was then incubated at the appropriate

temperature (Table 2.22) in a shaker for 1 hour. The tube was centrifuged for 4 minutes at 5000 g to gently pellet cells and 700 µL of supernatant was discarded, the pellet was then suspended in the remaining volume. Meanwhile a selection plate containing the appropriate antibiotics was heated at the appropriate temperature (Table 2.22), streaked with 30 µL of culture under sterile conditions, and put in the oven overnight at the appropriate temperature (Table 2.22), while the remaining cell culture was stored in 4°C.

*Table 2.22 Heat shock time and incubation temperature of the E. coli cell lines used.*

| Cell line name | Heat shock time | Incubation temperature |
|---|---|---|
| BL21 (DE3) Competent Cells | 45s | 37°C |
| Origami 2 (DE3) *pLysS* Competent Cells | 30s | 37°C |
| Shuffle T7 Express *lys Y* Competent Cells | 30s | 30°C |
| Lemo21 (DE3) Competent Cells | 10s | 37°C |

### 2.2.5 Induction of *E. coli* transformed cells with IPTG

50 mL of room-temperature LB medium were put in a 250 mL flask to which the appropriate amount of antibiotics was added. The flask was then inoculated with a single colony from a previously streaked LB agar plate containing the appropriate antibiotics, the flask was then incubated overnight in a shaker (250 rpm) at the appropriate temperature (Table 2.22). The next day, 25 mL of the bacterial culture were used to inoculate 500 mL of LB medium with no antibiotics added. The flask was incubated in a shaker (250 rpm) at the appropriate temperature (Table 2.22) for around 2 hours, then 750 µL of culture were taken to measure the absorbance using a

spectrophotometer. When the absorbance ($O.D._{600}$) reached a value between 0.4 and 0.8, the culture was induced with a 1 mM IPTG solution.

A 1.5 mL sample was taken right after induction and centrifuged 5 minutes at 18000 g to pellet the cells, the supernatant was removed, and the cells were resuspended in 200 µL lysis buffer (section 2.1.4). The tube was then stored at -20°C.

### 2.2.6 Measurement of cell culture O.D. using a spectrophotometer

Bacterial liquid culture absorbance was measured using Biowave DNA Life Science Spectrophotometer (WPA). 600nm absorbance was used to determine whether the culture was in the exponential phase.

### 2.2.7 Preparation of glycerol stock of transformed *E. coli* competent cells

500 µL of transformed *E. coli* competent cells were mixed with 500 µL of 50% v/v glycerol in a 2 mL tube and gently mixed. The glycerol stock was at -80°C. To recover bacteria from the glycerol stock, a sterile loop was used to scrape some cells and to streak the bacteria onto a LB agar plate containing the appropriate antibiotics; the plate was then incubated overnight at the appropriate temperature.

### 2.2.8 Sonication of bacterial cultures

A sonicator (a machine that uses ultrasound to cleave cell membranes) was used to perform four 20-second cycles intermixed with 30 seconds of cooling on ice. For small volumes sonication time was reduced, and the cooling time increased to reduce sample heating.

### 2.2.9 Isolation of inclusion bodies from *E. coli* cells induced with IPTG

Induced *E. coli* cells were centrifuged for 5 minutes at 18000 g, and the cell pellet was resuspended in PBS followed by a second centrifugation for 5 minutes at 20000 g, the supernatant was removed afterwards. The pellet was then resuspended in cold solution 1 (section 2.1.4) and incubated on ice for 10 minutes mixing gently, occasionally. 8 mM $MgCl_2$ and DNase 10 µg/mL were added to the solution and it was then incubated on ice for 10 minutes and centrifuged for 10 minutes at 20000 g at 4°C. The pellet was resuspended in 200 µL of solution 1 and, with the supernatant, was analysed by SDS-PAGE electrophoresis.

### 2.2.10 Preparation of polyacrylamide SDS-PAGE gels

The gel moulds were prepared by combining a Mini-PROTEAN short plate (Bio-Rad) and a Mini-PROTEAN spacer plate (Bio-Rad) to form the gel chamber. The two glasses were clamped with the plastic press, making sure that the bottom was leak proof by using a soft rubber mat as a plug. The resolving gel and the stacking gel solutions were prepared following Table 2.23 (volumes for two gels for each), using freshly made APS:

*Table 2.23 SDS-PAGE gel components.*

| **Reagent** | **Resolving gel 10%** | **Stacking gel 10%** |
|---|---|---|
| Water | 5.9 mL | 4.1 mL |
| 30% acrylamide solution | 5.0 mL | 1.0 mL |
| Resolving gel buffer, 1.5 M Tris, pH 8.8 | 3.8 mL | - |
| Stacking gel buffer, 0.5 M Tris, pH 6.8 | - | 750 µL |
| 10% SDS | 150 µL | 60 µL |
| 10% APS | 150 µL | 60 µL |

8 µL of TEMED were added per 15 mL of resolving gel solution and the solution was immediately poured to up to one centimetre from the glass chamber top, the remaining space was filled with water. The gel was let set for at least 30 minutes and then the water was removed, then 8 µL of TEMED were added to the stacking gel solution and it was poured to the top of the glass chamber and the comb was inserted. The gel was let set for at least 30 minutes, then the glass chamber was removed from the clamp and rinsed with water. The comb was removed, and the wells were rinsed as well. The prepared gels were either used or wrapped with a water-soaked paper towel and cling film around it and then stored in the fridge for up to a month.

### 2.2.11 Sample preparation for SDS-PAGE

1 volume of 2× Laemmli buffer (section 2.1.4) per volume of sample and 175 mM DTT were mixed, then 1 volume of sample was added, and the solution was boiled for 5 minutes.

### 2.2.12 Electrophoresis of proteins on SDS-PAGE gel

A gel "sandwich" was placed into each side of the electrode assembly, making sure that the short glass plates faced inwards, otherwise a plastic dam could be used on the other side. The electrode assembly containing the plates was placed in the supporting frame, clipped in place and then the supporting frame was placed in the tank. The inner tank was fully filled with fresh 1× TGS buffer (section 2.1.4) making sure that the top of the gel was covered, while the outer tank was filled half way. The samples were inserted using a pipette with thin tips, then the tank was closed, and the electrophoresis was run at 110 V for around 45 minutes. Once the run finished, the power was turned off, and

the glass chamber containing the gel was removed. Using a scalpel, the short plate was removed, the stacking gel was cut from the running gel and discarded and finally it was rinsed with water.

### 2.2.13 SDS-PAGE gels staining

#### 2.2.13.1 Microwave Coomassie Blue G-250 staining

The gel was transferred to a plastic tray and 20 mL of Coomassie brilliant blue stain (Thermo Fisher Scientific) solution was poured on it, allowing it to move freely in the tray. It microwaved for 10 to 20 seconds at maximum power, taking care not to boil the solution. The gel was then removed from the microwave and gently rotated for 5 to 10 minutes. Finally, the gel was rinsed twice in water.

#### 2.2.13.2 SimplyBlue SafeStain staining

The gel was rinsed 3 times for 5 minutes each with 100 mL deionized water to remove SDS and buffer salts, it was then stained with around 20 mL SimplyBlue SafeStain (Thermo Fisher Scientific) for 1 hour at room temperature with gentle shaking. After this time, the gel was washed with 100 mL of water for 1 hour.

#### 2.2.13.3 InstantBlue staining

The gel was transfer directly into 20 ml of InstantBlue (Expedeon) staining solution after the electrophoresis, making sure that it moves freely. The gel was stained for 15 minutes at room temperature with gentle shaking.

### 2.2.14 SDS-PAGE gel scanning using calibrated imaging densitometer model GS-800

All gels were scanned using the Bio-Rad calibrated imaging densitometer model GS-800 (Bio-Rad), coupled with the program Quantity One (Bio-Rad). Images were modified to increase the sharpness and contrast of the protein bands and then saved and printed.

### 2.2.15 Preparation of agarose gel

To prepare a 1% agarose gel, 1% w/v agarose and 1× TAE buffer were mixed in a flask. The solution was heated in a microwave until all the agarose crystals were melted then 0.5 µL ethidium bromide (EtBr) (10 mg/mL) was added per 10 mL of gel. The gel was let cool down or the temperature was decreased by placing the flask under water. In the meantime, the mould was tightly taped with masking tape and then the solution was poured in, the comb was then put in and the gel was let set. The comb was removed when ready to run the electrophoresis.

### 2.2.16 Agarose gel electrophoresis

The tank was filled with fresh 1× TAE buffer, the gel was then placed into the tank making sure that the buffer covers it. The samples were loaded into the wells, keeping one well for the marker. The electrophoresis was run at 110 V for around 45 minutes; the time can be modified depending on the length of the gel or to obtain greater separation between bands.

### 2.2.17 Agarose gel scanning

The UV lamp was heated for 10-15 seconds prior to the scanning (using MultiDoc-It imaging system, UVP) and then the gel was placed onto the UV plate. The best position

and focus for the gel were found using the “Preview” setting in the camera menu. A picture was taken, adjusting the capture time to best highlight the bands. The gel was then removed, and the UV surface cleaned with water and dried.

### 2.2.18 Protein purification

#### 2.2.18.1 Purification using Capturem™ His tagged purification miniprep kit

A Capturem™ His tagged purification miniprep kit (Takara) was used. 400 µL xTractor Buffer was added to a spin column in a collection tube, to equilibrate the column. The column was centrifuged at 11,000 g for 1 minute at room temperature and the flow through was discarded with the collection tube, the column was then placed in a new collection tube. Around 500 µL of sonicated lysate was loaded onto the equilibrated spin column and centrifuged at 11,000 g for 1 minute at room temperature. The lysate flow through was saved for protein analysis and the spin column was transferred to a new collection tube to which 300 µL of Wash Buffer were added. The tube was centrifuged at 11,000 g for 1 minute at room temperature and again the flow through was saved for protein analysis and the spin column was transferred to a new collection tube. 300 µL of Elution Buffer were added to the spin column which was then centrifuged at 11,000 g for 1 minute at room temperature. The flow through was used to analyse the protein purification with an SDS-PAGE gel.

#### 2.2.18.2 Purification using a nickel affinity cartridge

The cartridge was equilibrated with at least 25 mL of binding buffer. Meanwhile the sample was sonicated, centrifuged and filtered through a 0.45 µm filter immediately before the application to the cartridge (Nickel affinity cartridge, ABT) and then inserted

into it. The cartridge was then washed with 50 mL of binding buffer and the flow through was sampled to measure the $O.D._{280}$, washing continued until the O.D. stabilised. 25 mL of elution buffer were added to the cartridge. The flow through was saved for SDS-PAGE analysis. Before storage, the cartridge was washed with 25mL of 20% v/v ethanol and then kept in the fridge. All buffers were provided with the kit.

#### 2.2.18.3 Purification using nickel affinity magnetic beads

The sample was centrifuged, and the supernatant discarded, the pellet was then resuspended in 200 µL of binding buffer (section 2.1.4). The magnetic beads bottle was shaken to homogenize the suspension and 200 µL of solution were pipetted in a clean tube. The beads were separated with a magnet and the preservative was removed from the beads (Nickel affinity magnetic beads, ABT). 500 µL of binding buffer were added to the beads and the tube was mixed to form a homogeneous suspension. Again, the beads were separated with a magnet and the supernatant was discarded, 1 mL of sample was then added to the beads and the suspension was mixed for 1 hour at 4°C. Once done, the beads were separated with a magnet and then washed and vortexed with 500 µL of washing buffer (section 2.1.4). The washing step was repeated twice. 100 µL of elution buffer (section 2.1.4) were added to the beads and mixed for 10 minutes. The beads were separated with a magnet and the elution fraction was collected and stored on ice.

### 2.2.19 Western blot

Transfer buffer was prepared and kept in the freezer while preparing the electrophoresis membrane by soaking it in methanol for around 10 seconds. A freshly run SDS-PAGE gel was washed in water and then in transfer buffer for 15 minutes, a WB

membrane was also washed in transfer buffer for 15 minutes, while the WB sponges were soaked in cold transfer buffer. The WB electrophoresis sandwich was prepared starting from the negative charged side and stacking a sponge, the previously produced gel (section 2.2.16), a membrane (section 2.1.9) and finally a second sponge.

An ice pack was added in the western blot chamber which was then filled with cold transfer buffer to the top. The western blot was run at 100V for 30 minutes; meanwhile, 25 mL of TBS-T BSA 3% w/v was prepared. Once the western blot finished running, the right top angle of the membrane was cut to be able to distinguish the protein face, and the membrane was blocked with BSA for 1 hour at room temperature. Afterwards, the membrane was rinsed in TBS-T and the primary antibody solution was prepared in a 50 mL falcon tube (section 2.1.2). The membrane was put into the tube that was then put in the cold room overnight on a shaker. The day after the primary antibody solution was removed and the membrane was washed in TBS-T 6 times for 5 minutes each time. The ECL reagents were normalised to room temperature while the secondary antibody solution was prepared in a 50 mL falcon tube (section 2.1.2). ECL marker and S-protein were added together to the secondary antibody solution and the membrane was incubated with the secondary antibody solution at room temperature, shaking for 1 hour. Once completed, the secondary antibody solution was discarded, and the membrane was washed in TBS-T 6 times for 5 minutes each time. 250 µL of 1:1 ECL reagents solution per membrane was prepared, liquid was removed from the membrane and it was then placed between plastic sheets. The ECL reagents solution was then put on top of it and the plastic cover was closed trying to remove most of the

bubbles and finally the sides were sealed with heat. The ECL reagents solution was activated by putting the membrane in the oven at 37 °C for 5 minutes.

### 2.2.20 *In vitro* transcription

*In vitro* transcription was performed using two different kits from two different manufacturers.

#### 2.2.20.1 Producing *in vitro* transcription using mMessage mMachine

The mMessage mMachine system (ThermoFischer scientific) was used to produce the transcription. Reagents (listed in Table 2.24) were thawed and vortexed at room temperature except for the RNA polymerase Enzyme Mix that was kept on ice. Once thawed, the ribonucleotides (2× NTP/CAP) were stored on ice, but the transcription reaction was assembled at room temperature. The 10× Reaction Buffer was added after the water and the ribonucleotides.

*Table 2.24 mMessage mMachine reaction components.*

| **Component** | **Volume** |
|---|---|
| Nuclease-free Water | to 20 µL |
| 2× NTP/CAP | 10 µL |
| 10× Reaction Buffer | 2 µL |
| Linear template DNA | 0.1–1 µg |
| Enzyme Mix | 2 µL |

The solution was mixed thoroughly and then centrifuged briefly, it was then incubated at 37°C, for 2 hours.

#### 2.2.20.2 Producing *in vitro* transcription using Riboprobe in vitro transcription system

The Riboprobe in vitro transcription system (Promega) was also used to produce the transcription. The components listed in Table 2.25 were added at room temperature in the order listed.

*Table 2.25 Riboprobe reaction components.*

| **Reagent** | **Volume** |
|---|---|
| Transcription Optimized 5× Buffer | 4 µL |
| DTT, 100mM | 2 µL |
| Recombinant RNasin® Ribonuclease Inhibitor | 20–40 units |
| rATP, rGTP and rUTP (2.5mM each) | 4 µL |
| 100µM rCTP (diluted from stock) | 2.4 µL |
| Linearized template DNA (0.2–1.0mg/ml in water or TE buffer) | 1 µL |
| SP6, T3 or T7 RNA Polymerase | 15–20 units |
| Final volume | 20 µL |

The reaction was incubated for 1 hour at 37°C.

### 2.2.21 *In vitro* translation

#### 2.2.21.1 Producing *in vitro* translation using Rabbit reticulocyte lysate system

The Rabbit reticulocyte lysate system (Promega) was used to produce *in vitro* translation. Reagents listed in Table 2.26 were removed from storage and allowed to thaw slowly on ice. Meanwhile the template mRNA was denatured at 65°C for 3 minutes, and immediately cooled in an ice-water bath to remove secondary structures. The following components were assembled in a 0.5ml polypropylene microcentrifuge tube.

*Table 2.26 Rabbit reticulocyte lysate reaction components.*

| **Reagent** | **Volume** |
|---|---|
| Rabbit Reticulocyte Lysate | 35 µL |
| Amino Acid Mixture Minus Leucine, 1mM | 0.5 µL |
| Amino Acid Mixture Minus Methionine, 1mM | 0.5 µL |
| RNasinR Ribonuclease Inhibitor (40u/µL) | 1 µL |
| RNA substrate in water (1µg/µL) | 2 µL |
| Transcend™ Biotin-Lysyl-tRNA or FluoroTect™ tRNA GreenLys tRNA | 1–2 µL |
| Nuclease-Free Water to a final volume of | 50 µL |

The translation reaction was then incubated at 30°C for 90 minutes.

### 2.2.22 Sequencing of vector and DNA primers

All sequencing was outsourced to Source BioScience (Nottingham, UK). All samples were sent in a 5 µL per-reaction volume at a concentration of 100 ng/mL. Samples were analysed by Source BioScience using M13 primers, using either forward or forward and reverse. Resulting sequences were analysed using *FinchTV* chromatogram viewer.

### 2.2.23 Homogenisation of insect samples

Insect samples were put into a 2mL tube containing the same buffer to be used in the procedure that the sample is being homogenised for. The homogeniser was let run in a 2mL tube filled with the same buffer to remove air bubbles. Each sample was homogenised with 20 seconds bursts, until the solution became clear of debris. Three water baths and an EtOH step were used to clean and sterilise the homogeniser.

### 2.2.24 Phenol/chloroform total DNA and RNA extraction

Each freshly homogenised sample was centrifuged to pellet the debris, and the supernatant was transferred in a new tube. 1 volume of acid Phenol (pH 4.3) was added and vortexed to thoroughly mix the solution. The tube was then centrifuged for 5

minutes at maximum speed. The superior phase was carefully pipetted out, trying not to touch or disturb the interface. In cases when the solution did not form interfaces, 1 volume of chloroform: isoamyl alcohol in a 9:1 ratio was added and vortexed and centrifuged again. After, 1 volume of chloroform: isoamyl alcohol was added, vortexed and centrifuged, then the upper phase was pipetted out and stored in -20°C freezer or used immediately.

### 2.2.25 Total RNA extraction with ZR Tissue and Insect RNA MicroPrep

The extraction can be performed using total DNA and RNA extracted with phenol/chloroform or using a freshly homogenised sample, which needs to be previously centrifuged to separate the debris. The total RNA extraction was performed using the ZR Tissue and Insect RNA MicroPrep (Zymo Research). 400 µL of sample was transferred to a Zymo-Spin IIIC column in a collection tube and centrifuged for 30 seconds at maximum speed. The flow through was mixed well with 400 µL of pure ethanol and then transferred to a Zymo-Spin IC column in a collection tube, the column was then centrifuged for 30 seconds and the flow through discarded. 400 µL of RNA Prep Buffer was added to the column, the tube was centrifuged for 30 seconds and the flow through discarded. 700 µL of RNA Wash Buffer was added to the column, the tube was centrifuged for 30 seconds and the flow through discarded. 400 µL of RNA Wash Buffer was add to the column and the tube centrifuged for 2 minutes. Finally, the column was transferred into an RNase free tube to which 15 µL of cold DNase/RNase free water was added and the tube was centrifuged for 30 seconds. The eluted RNA was stored in a -80°C freezer or used immediately.

### 2.2.26 cDNA synthesis

Two different reverse transcriptase enzymes were used to perform the cDNA synthesis.

#### 2.2.26.1 PCR using the Transcriptor reverse transcriptase

The first enzyme that was used was the Transcriptor reverse transcriptase (Sigma-Aldrich). The components listed in Table 2.27 were added to a nuclease-free microcentrifuge tube:

*Table 2.27 Transcriptor reverse transcriptase reaction components 1.*

| **Reagent** | **Volume** |
|---|---|
| RNA template | 2 µL |
| Reverse primer | 3 µL |
| Water | 8 µL |

The tube was incubated at 65°C for 10 minutes to denature RNA's secondary structures and then put immediately on ice for 2 minutes. Then the components listed in Table 2.28 were added:

*Table 2.28 Transcriptor reverse transcriptase reaction components 2.*

| **Reagent** | **Volume** |
|---|---|
| Transcriptor RT reaction buffer | 4 µL |
| dNTPs mix (10 mM) | 2 µL |
| Transcriptor reverse transcriptase | 0.5 µL |

The solution was vortexed and centrifuged, then incubate in the PCR machine with protocol in Table 2.29. Finally, the Transcriptor reverse transcriptase was inactivated by heating to 85°C for 5 minutes followed by cooling the tube on ice for 5 minutes.

*Table 2.29 Transcriptor RT thermal cycler program*

| Time | Temperature |
|---|---|
| 30 minutes | 25°C |
| 30 minutes | 45°C |
| 30 minutes | 55°C |

### 2.2.26.2 PCR using the SuperScript III reverse transcriptase

The second enzyme that was used was the SuperScript III reverse transcriptase (Thermo Fisher Scientific). The components listed in Table 2.30 were added to a nuclease-free microcentrifuge tube:

*Table 2.30 SuperScript III reverse transcriptase reaction components 1.*

| **Reagent** | **Volume** |
|---|---|
| random primers | 50–250 ng |
| total RNA | 10 pg–5 µg |
| 10 mM dNTP mix | 1 µL |
| Water | 14 µL |

Then the mixture was heated to 65°C for 5 minutes and incubate on ice for 1 minute. The tube was then centrifuged, and the components listed in Table 2.31 were added:

*Table 2.31 SuperScript III reverse transcriptase reaction components 2.*

| **Reagent** | **Volume** |
|---|---|
| 5× First-Strand Buffer | 4 µL |
| 0.1 M DTT | 1 µL |
| SuperScript™ III RT (200 units/µL) | 1 µL |

The solution was mixed by pipetting gently up and down and then put in the PCR machine with protocol in Table 2.32.

*Table 2.32 Superscript RT thermal cycler program*

| Temperature | Duration | Cycles |
|---|---|---|
| 25°C | 5 minutes | 1 |
| 50°C | 60 minutes | 1 |
| 70°C | 15 minutes | 1 |

### 2.2.27 Polymerase chain reaction (PCR) conditions

The PCR buffer, the dNTP mix and the primers were thawed at room temperature and then vortexed. A reaction mix was created following Table 2.33, the reaction mix volume can be modified as needed. The reaction mix was kept on ice at all times.

*Table 2.33 PCR reaction mix*

| Component | Volume | Final concentration |
|---|---|---|
| 10× PCR buffer | 2.5 µL | 1× |
| dNTP mix (10 µM each) | 0.5 µL | 200 µM of each dNTP |
| Primer A | 0.125 µL | 0.5 µM |
| Primer B | 0.125 µL | 0.5 µM |
| HotStarTaq DNA polymerase | 0.125 µL | 2.5 units/reaction |
| DNA | Variable | ≤1 µg/100 µl reaction |
| Water | To 25 µL | |

Samples were defrosted and vortexed, the master mix was divided into tubes to which the samples were added. The tubes were then put in a thermal cycler and the appropriate program was selected (programs listed in the next paragraphs).

After the completion of the thermal cycler's program, samples were stored in the fridge.

Below are the temperatures and the duration of each PCR step for the 7 different reaction types used in the experiments of this thesis. All annealing temperatures were modified accordingly to the primers' melting temperature.

#### 2.2.27.1 General hot-start touchdown PCR

Conditions in Table 2.34 were used to elongate a cDNA fragment created using random primers, which produces a set of DNA sequences. The touchdown step reduces the temperature one degree per cycle, insuring a perfect annealing between target sequence and the primers.

*Table 2.34 General hot-start touchdown PCR.*

| **Temperature** | **Duration** | **Cycles** |
|---|---|---|
| 95°C hot-start | 15 minutes | 1 |
| 65°C to 45°C touchdown | 1 minute per degree | 20 |
| 95°C | 30 seconds | 40 |
| 55°C | 30 seconds | |
| 72°C | 30 seconds | |
| 72°C | 2 minutes | 1 |

#### 2.2.27.2 Standard PCR

Conditions in Table 2.35 were used to elongate a DNA segment created during previous PCR, either to increase its concentration or to produce a new batch.

*Table 2.35 Standard PCR.*

| **Temperature** | **Duration** | **Cycles** |
|---|---|---|
| 95°C hot-start | 15 minutes | 1 |
| 95°C | 30 seconds | 35 |
| 59°C | 30 seconds | |
| 72°C | 45 seconds | |
| 72°C | 5 minutes | 1 |

#### 2.2.27.3 T7 PCR

Conditions in Table 2.36 were used to elongate the already formed DNA strands using the T7 primers, to create probes including the T7 sequences.

*Table 2.36 T7 PCR.*

| Temperature | Duration | Cycles |
|---|---|---|
| 95°C hot-start | 15 minutes | 1 |
| 95°C | 30 seconds | 35 |
| 67°C | 30 seconds | |
| 72°C | 1 minute | |
| 72°C | 5 minutes | 1 |

#### 2.2.27.4 MEGAscript T7 PCR

Conditions in Table 2.37 were used to perform the last step of the production of the probes containing the T7 tails, using the MEGAscript kit.

*Table 2.37 MEGAscript T7 PCR.*

| Temperature | Duration | Cycles |
|---|---|---|
| 94°C | 3 minutes | 1 |
| 93°C to 45°C touchdown | 1 minute per degree | 48 |

#### 2.2.27.5 M13 HotStar Plus PCR

Conditions Table 2.38 were used to anneal the M13 sequences to an existing DNA fragment. The M13 primers are specifically made for Sanger sequence verification, to check for the DNA sequences quality.

*Table 2.38 M13 HotStar Plus PCR.*

| Temperature | Duration | Cycles |
|---|---|---|
| 95°C hot-start | 15 minutes | 1 |
| 95°C | 30 seconds | 28 |
| 57°C | 30 seconds | |
| 72°C | 1 minute | |
| 72°C | 5 minutes | 1 |

### 2.2.28 Extraction of DNA from agarose gel using QIAEX II gel extraction kit

The QIAEX II gel extraction kit (QIAGEN) was used to extract DNA from agarose gels. Gel bands were excised from the agarose gel and put in a 1.5 mL tube to which 3 times the volume of the gel in Buffer QX1 was added. Meanwhile the matrix of Qiaex II was resuspended by vortexing the tube for 30 seconds and then 10 µL of it were added to the sample tube. The mix was incubated at 50°C for 10 minutes, vortexing every two minutes to speed up the solubilisation of the gel. The sample was then centrifuged for 1 minute to form a pellet and the supernatant was removed using a vacuum machine. The pellet was washed with 500 µL of Buffer QX1, resuspended by vortexing and then centrifuged for 1 minute. The supernatant was again removed with a vacuum machine. The pellet was washed twice with 500 µL of Buffer PE, resuspended by vortexing, and then centrifuged for 1 minute. The supernatant was again removed with a vacuum machine. The pellet was air dried until it became white then it was diluted in 15 µL of Elution Buffer and incubate at room temperature for 5 minutes. Finally, the tube was centrifuged for 1 minute and the DNA-containing supernatant was pipetted into a new tube.

### 2.2.29 Ligation of DNA into pCR4-TOPO TA

TOPO vector is supplied as 10 ng/µL plasmid DNA in a buffer, its components are listed in Table 2.39.

*Table 2.39 pCR 4-TOPO buffer composition*

| **pCR 4-TOPO** |
|---|
| 50% glycerol |
| 50 mM Tris-HCl, pH 7.4 |
| 1 mM EDTA |
| 2 mM DTT |
| 0.1% Triton X-100 |
| 100 µg/mL BSA |
| 30 µM phenol red |

The reagents were used in the order listed in Table 2.40.

*Table 2.40 Ligation reaction components.*

| **Reagent** | **Volume** |
|---|---|
| Fresh PCR product | 0.5–4 µL |
| Salt Solution (1.2 M NaCl, 0.06 M $MgCl_2$) | 1 µL |
| Water | add to a total volume of 5 µL |
| TOPO vector | 1 µL |
| Final Volume | 6 µL |

The reaction was mixed and incubated for 5 minutes at room temperature; it was then put onto ice and used to transform *E. coli* competent cells.

### 2.2.30 Isolation of plasmid DNA from bacteria using Zyppy plasmid miniprep kit

The 'miniprep' isolation is a procedure to extract plasmid DNA from bacteria. The miniprep preparation was performed using the Zyppy plasmid miniprep kit (Zymo Research) at room temperature. 600 µL of bacterial culture were put in a 1.5 mL tube to which 100 µL of 7× Lysis buffer were added; the solution was mixed by inverting the

tube 4-6 times and incubated for 1-2 minutes. Then 350 µL of cold neutralisation buffer were added and mixed thoroughly and the tube was centrifuge for 2-4 minutes. The supernatant was then transferred into a Zymo-spin IIN column in a collection tube and centrifuged for 15 seconds. The flow through was discarded and 200 µL of Endo-wash buffer were added to the column and centrifuged for 1 minute then 400 µL of Zyppy wash buffer were added to the column and centrifuged for another minute. The column was transferred into a clean 1.5 mL tube and 30 µL of Zyppy elution buffer were added directly to the column matrix and incubated for 1 minute at room temperature, then the tube was centrifuged for 30 seconds to elute the plasmid DNA. The eluted plasmid was stored in the freezer.

### 2.2.31 T7 primers ligation using MEGAscript T7

All reagents listed in Table 2.41 were thawed at room temperature, except for the DNA polymerase and the 4 ribonucleotide solutions that were kept on ice. All solution except for the DNA polymerase, were vortexed until completely in solution. The transcription reaction was assembled at room temperature and the 10× Reaction Buffer was added after the water and the ribonucleotides were already in the tube. The amounts given in table 2.35 are for a single 20 µL reaction.

*Table 2.41 MEGAscript T7 reaction components 1.*

| Component | Concentration | Amount |
|---|---|---|
| Nuclease-free water | | To 20 µL |
| ATP solution | 75 mM | 2 µL |
| CTP solution | 75 mM | 2 µL |
| GTP solution | 75 mM | 2 µL |
| UTP solution | 75 mM | 2 µL |
| 10× Reaction buffer | 1× | 2 µL |
| Linear template DNA | Variable | 0.1-0.2 µg |
| Enzyme mix | 4 units | 2 µL |

The solution was mixed thoroughly and incubated at 37°C overnight. The day after 1 µL of TURBO DNase was added and the solution was incubated for 15 minutes at 37°C. The tube was put in the PCR machine using protocol in Table 2.37. The dsRNA was precipitated by adding the reagents listed in Table 2.42, per 20 µL of solution:

*Table 2.42 MEGAscript T7 reaction components 2.*

| Reagent | Concentration | Volume |
|---|---|---|
| RNase free water | | 280 µL |
| Sodium acetate (3 M, pH 5.2) | | 30 µL |
| Ethanol | | 650 µL |

The solution was placed at -20°C overnight or in -80 °C for 1 hour (adding double the EtOH), then it was centrifuged at 15500g for 30 minutes at 4 °C. Supernatant was removed and the pellet was washed with 70% v/v ethanol, after which the pellet was air dried and dissolved in 20 µL injection buffer (Table 2.5 Buffers). The dsRNA was stored at -20 °C.

### 2.2.32 Measurement of double stranded RNA concentration

dsRNA concentration was measured using Biowave DNA Life Science Spectrophotometer (WPA). The ABS ratio measurement was used to calculate the

samples' absorbance at 260nm and 280nm. This system is used because nucleic acids have their maximum absorbance at 260nm, while proteins have theirs at 280nm this method is used to produce a ratio that can be used to determine the purity of the sample. Both sample dilution and RNA nature of the sample were set, so that the machine calculated the total concentration of dsRNA.

### 2.2.33 Rearing of *Goniozus legneri*

*G. legneri* cultures were kept at 27°C and were reared and maintained in glass tubes each containing one *Corcyra cephalonica* (Lepidoptera: Pyralidae) moth larva as host. The humidity in the culture room was maintained at 25% R.H. by evaporation from a water bath. Adult *G. legneri* wasps were not fed, and new tubes containing one *G. legneri* wasp and one caterpillar were prepared twice per week to maintain the population.

### 2.2.34 Injections into *Goniozus legneri*

The possibility of injecting solutions directly into the body of developing *G. legneri* wasps was explored using a methodology like the one used by Lynch and Desplan (Lynch and Desplan 2006). Glass needles were prepared by pulling calibrated glass capillary tubes over a flame. The pulled glass needle was then connected to a 10 µL syringe with a flexible plastic tube.

Larvae where injected once they moved away from the host (which they do once they have completed feeding and prior to spinning a cocoon in which to pupate), as they will not reattach to the host once removed. Larvae were positioned under a microscope and kept still with the help of tweezers and the tip of the needle was positioned in the centre

of the larva and a slight pressure was put onto them until the needle pierced the integument.

Pupae were collected on the second day after they produced the cocoon, the cocoon was removed using tweezers making sure not to hurt the insect. The injection was performed on the abdomen.

### 2.2.35 Rearing of *Corcyra cephalonica*

*Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae) was reared and maintained in glass mason jars containing a mix of corn meal, wheat bran and glycerol utilised as a food source by the caterpillars. Moths were put in the jars to lay eggs and once they hatched, the caterpillars grew until new adult moths emerged to start the cycle anew. The cultures were kept at 27°C with air humidity maintained at 25% RH.

### 2.2.36 Injections in *Corcyra cephalonica*

Each caterpillar was weighed and selected if the weight was between 30 and 40 mg, it was then individually put in a glass tube, and the tube was plugged with a cotton ball wrapped in gauze. The tubes were numbered consequentially and then a single *Goniozus legneri* wasp was added to each one of them. The tubes were left in the culture room checking for the presence of eggs to confirm that the wasp stung the caterpillar and started laying. Injections were performed using a Hamilton 10 µL syringe, model 1701 SN SYR, cemented NDL, custom gauge (33), custom point style (4), angle (45), custom needle length (30mm). Injections were performed under the microscope, with the help of a pair of tweezers to keep the host still. The injection site was not constant,

instead it was changed according to the disposition of the larvae on the host. Injections were performed on the second day of larval state.

### 2.2.37 Rearing of *Gryllus bimaculatus*

*G. bimaculatus* crickets were obtained from Monkfield Nutrition Ltd. (Royston, Cambridgeshire, UK) at the 5$^{th}$ instar and reared at 28°C. Crickets were put into plastic containers at arrival and were given pelleted chicken food and free access to water. Egg carton was added to provide shelter for the crickets. Water and food were provided *ad libitum*.

### 2.2.38 Injections in *Gryllus bimaculatus*

*G. bimaculatus* crickets were chilled in the freezer for 10 minutes in a plastic box and then put on ice. A cold pack was used as "operating surface" on which one cricket at a time was positioned. The wrist of a nitrile glove was cut and then the side was cut to form a strip, the strip was cut in half and a small hole was created in its centre. This nitrile strip was used to keep the crickets still and in contact with the cold surface, with the hole positioned on their abdomen to highlight the third and fourth abdominal segment to allow for the injection between them. The solution was injected into the abdominal body cavity and then the cricket was moved in another plastic box at room temperature, once the crickets started recovering and moving, they were transferred back to the warm room.

# Chapter 3 Expression, purification, and optimisation of the production of Royalactin

## 3.1 Introduction

Eusociality can be found in some insects such as some bees and wasps and all ants, and termites. One of the defining features of such advanced sociality is reproductive division of labour, often manifest as separate castes carrying out different roles. It is common to find different phenotypes, which each egg can potentially develop into, in a process described as environmentally driven phenotypic plasticity (Maleszka, 2008). This means that the variations in the phenotypes are not directly caused by differences in the DNA sequence, but instead by modifications in gene expression during development. In the honey bee, *Apis mellifera*, for example, the phenotypic differences between workers and queens do not have a genetic basis but operate in response to environmental stimuli. Specifically, they are induced by the quantity of royal jelly that the larvae are fed during development (Wilson, 1971, Wilson, 1979). During their larval stage, worker bees are fed royal jelly for three days, which is then supplemented by a mix of pollen, honey (in addition to royal jelly) for the remaining three days (Chapter 1, section 1.1). Queen bee larvae, in contrast, are fed exclusively on royal jelly for a total of 5 days throughout their entire larval stage, after which the cell will be capped, and they pupate. The adult queen is also fed royal jelly for her entire life. This difference in diet leads to the expression of two distinct adult female phenotypes.

Royal jelly must contain a set of substances or a single substance that causes changes in epigenetic modifications that are linked with caste-specific gene-expression patterns. It is still unknown if these changes are mediated by only one of royal jelly's constituents

or if the modifications are produced by multiple factors. In the 2011 paper entitled *Royalactin induces queen differentiation in honeybees'*, Kamakura (2011) provided evidence that the phenotypic changes, such as longevity, fecundity and increased body mass, were caused by a single protein, MRJP1, that he renamed Royalactin (Figure 3.1).

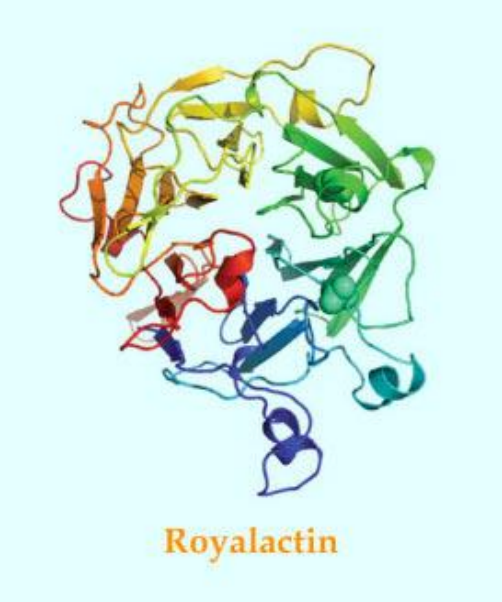



*Figure 3.1 Computational modelling prediction of the structure of Royalactin. Taken from Wan et al. (2018).*

Royalactin is a member of the Yellow gene family (Buttstedt et al., 2014, Ferguson et al., 2011), a family of genes that is conserved in all insects. Kamakura argued that it is possible that Royalactin can influence body mass, fecundity, and longevity in other insect species, beyond honey bees. Kamakura therefore tested the effects of Royalactin on *Drosophila melanogaster* larvae by adding to their diet either royal jelly devoid of Royalactin or royal jelly containing the protein and reported that Royalactin has a body enlarging effect (Kamakura, 2011). Kamakura also used *D. melanogaster* mutants to study the pathways involved in the modifications caused by Royalactin, the same author

used *D. melanogaster* mutants. Epidermal growth factor receptor (EGFR) mutants were not affected by dietary Royalactin, and did not show any body enlarging effect, suggesting that Royalactin possess a function similar to the epidermal growth factor.

Xin et al. (Xin et al., 2016) subsequently showed that feeding *Drosophila* with a diet containing 2.5% of royal jelly's proteins positively influenced lifespan, feeding, fecundity and the EGFR-mediated signalling pathway. Other studies have demonstrated an involvement of the EGFR pathway in phenotypic variation in animals, such as difference in body size or lifespan. Dabour et al. (2011) knocked down the expression of the EGFR gene in the cricket *Gryllus bimaculatus* using RNA interference during its development, resulting in a decrease in the size of the maturing adults. Alvarado et al. (2015) that ants can generate continuous size variations of workers body size by naturally inducing a genome-wide DNA methylation. They also showed how it was related specifically to the methylation of the EGFR gene. Detienne et al. (2014), showed how Royalactin extends the lifespan of *Caenorhabditis elegans* through the EGFR pathway by promoting the EGF signalling pathway.

The hypothesis underlying this chapter is that Royalactin could activate pathways in insects other than honey bees and *Drosophila*, as shown by Kamakura (2011). The aim was to express recombinant Royalactin, purify it and examine the effects of feeding it to further insect species.

This chapter first describes how recombinant Royalactin was produced in the bacterium *Escherichia coli*. A series of *E. coli* cell lines were tested, and the results are listed below in section 3.2.2. The chapter also reports the optimisation of, and the amendments to

the initial protocol and the approaches that were taken to purify the protein. Also described are the modifications to Kamakura's protein expression protocol that were made to be able to produce recombinant Royalactin.

## 3.2 Methods

### 3.2.1 Cloning of Royalactin

It was decided to produce recombinant Royalactin, as opposed to extracting the protein from royal jelly through ultracentrifugation. This is because expressing recombinant proteins using bacterial cells is generally a rapid and inexpensive way to produce the protein, it can also be easily scaled up to obtain high yields. In contrast, the ultracentrifugation approach to purify Royalactin not only has a lower yield but is also more expensive as it needs high quantities of royal jelly as source.

Royalactin's complementary DNA (cDNA) was cloned into pET28b vector (section 2.1.11) by Dr Reinhard Stöger (University of Nottingham). Construction of the Royalacin-pET28b expression vector replicated the strategy described by Kamakura (2011) paper's supplementary materials. Information of the cDNA sequence was obtained from GenBank (accession number GQ160518.1); it encodes Royalactin's mature protein excluding the signal peptide (1278 base pairs) (Table 3.1). The cDNA sequence was synthesised by GeneART Gene Synthesis (Thermo Fisher Scientific). The cDNA was cloned into a pET28b vector (Novagen), between the NdeI and SacI restriction sites within the vector's multiple cloning site. To ensure integrity of the construct, the cloned plasmid was sent to a commercial company, Source BioScience (Nottingham, UK), for sequencing, using the 'insert-integrity' primers listed in 2.1.11.1. The result showed a

single adenine base change at the non-coding-3' end of the cDNA sequence, outside the open reading frame (ORF): AGCTCGA**A**TTCGGA (marked in bold in this portion of the sequence). This point mutation does not affect the amino acid sequence of Royalactin; therefore, it was decided to use this construct for subsequent expression experiments.

The recombinant protein exposes a HIS-tag at the N-terminus, the HIS-tag is a sequence of 6 histidine that originates from the pET28b plasmid and is translated together with the protein. In the pET28b vector there are two sets of HIS-tag that flank the multiple cloning site on both sides. The C-terminus does not expose the HIS-tag as the stop codon is upstream of it.

*Table 3.1 Royalactin's characteristics.*

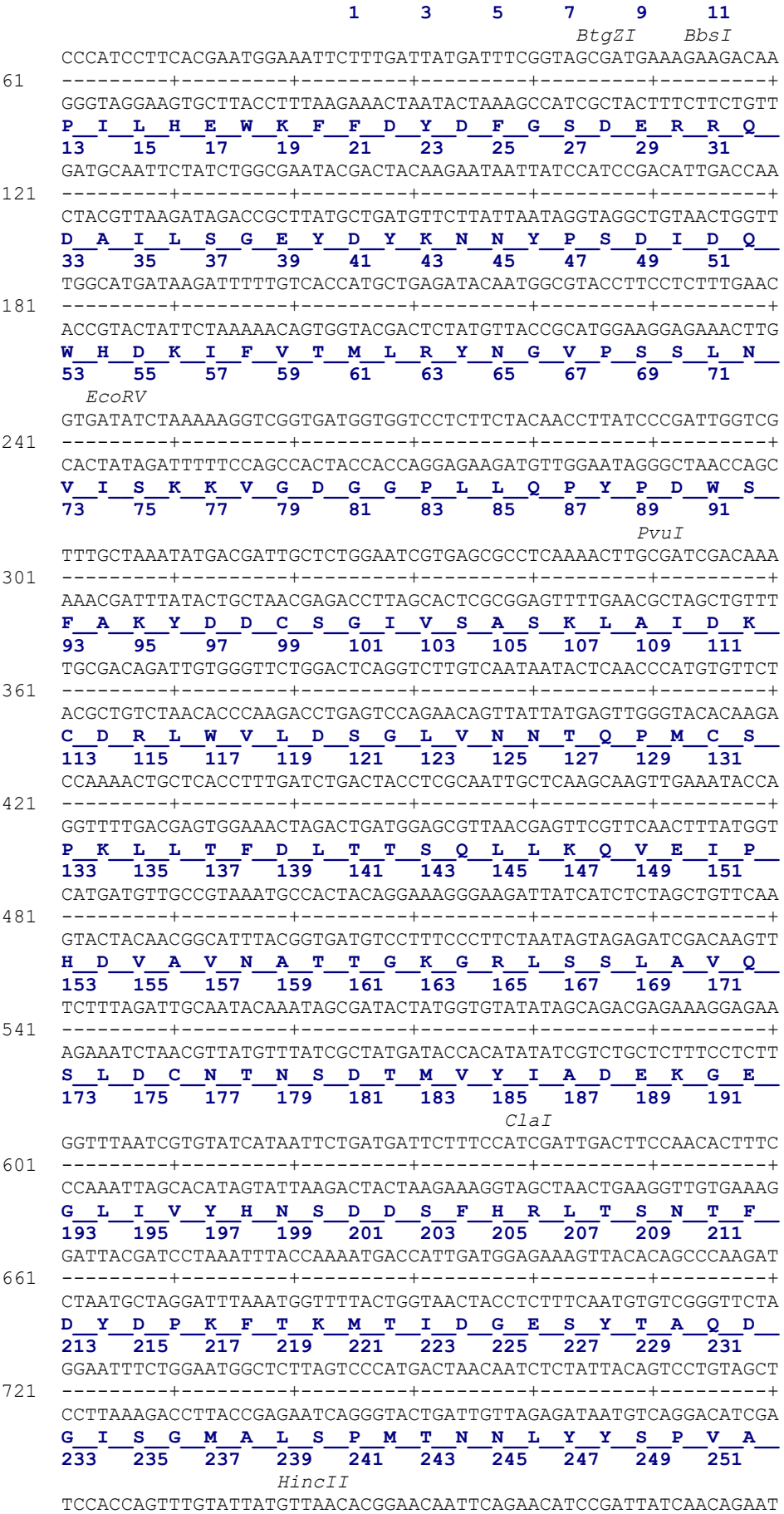
| Royalactin | Molecular weight | Amino acids | DNA sequence length |
|---|---|---|---|
| Synonyms: Mrjp1, major royal jelly protein 1 | 46860.56 kDa | 413 | 1278 bp |

All the cloning was performed before the beginning of my PhD work and is the only portion of this thesis that was not carried out by me: all the following experiments described here were performed by me only.

### 3.2.1.1 Royalactin DNA sequence

The DNA sequence encoding Royalactin is shown below. The amino acid sequence is shown in bold underneath the DNA sequence and the positions of recognition sites for restriction enzymes are indicated above it, in italics.

```
                  SacII
     TATTTCCAGGGAGCAGCCGCGGCCAACATTCTTCGAGGAGAGTCTTTAAACAAATCATTA
1    ---------+---------+---------+---------+---------+---------+
     ATAAAGGTCCCTCGTCGGCGCCGGTTGTAAGAAGCTCCTCTCAGAAATTTGTTTAGTAAT
                           N__I__L__R__G__E__S__L__N__K__S__L__
```


1 3 5 7 9 11
BtgZI BbsI
CCCATCCTTCACGAATGGAAATTCTTTGATTATGATTTCGGTAGCGATGAAAGAAGACAA
GGGTAGGAAGTGCTTACCTTTAAGAAACTAATACTAAAGCCATCGCTACTTTCTTCTGTT
P I L H E W K F F D Y D F G S D E R R Q
13 15 17 19 21 23 25 27 29 31
GATGCAATTCTATCTGGCGAATACGACTACAAGAATAATTATCCATCCGACATTGACCAA
CTACGTTAAGATAGACCGCTTATGCTGATGTTCTTATTAATAGGTAGGCTGTAACTGGTT
D A I L S G E Y D Y K N N Y P S D I D Q
33 35 37 39 41 43 45 47 49 51
TGGCATGATAAGATTTTTGTCACCATGCTGAGATACAATGGCGTACCTTCCTCTTTGAAC
ACCGTACTATTCTAAAAACAGTGGTACGACTCTATGTTACCGCATGGAAGGAGAAACTTG
W H D K I F V T M L R Y N G V P S S L N
53 55 57 59 61 63 65 67 69 71
EcoRV
GTGATATCTAAAAAGGTCGGTGATGGTGGTCCTCTTCTACAACCTTATCCCGATTGGTCG
CACTATAGATTTTTCCAGCCACTACCACCAGGAGAAGATGTTGGAATAGGGCTAACCAGC
V I S K K V G D G G P L L Q P Y P D W S
73 75 77 79 81 83 85 87 89 91
PvuI
TTTGCTAAATATGACGATTGCTCTGGAATCGTGAGCGCCTCAAAACTTGCGATCGACAAA
AAACGATTTATACTGCTAACGAGACCTTAGCACTCGCGGAGTTTTGAACGCTAGCTGTTT
F A K Y D D C S G I V S A S K L A I D K
93 95 97 99 101 103 105 107 109 111
TGCGACAGATTGTGGGTTCTGGACTCAGGTCTTGTCAATAATACTCAACCCATGTGTTCT
ACGCTGTCTAACACCCAAGACCTGAGTCCAGAACAGTTATTATGAGTTGGGTACACAAGA
C D R L W V L D S G L V N N T Q P M C S
113 115 117 119 121 123 125 127 129 131
CCAAAACTGCTCACCTTTGATCTGACTACCTCGCAATTGCTCAAGCAAGTTGAAATACCA
GGTTTTGACGAGTGGAAACTAGACTGATGGAGCGTTAACGAGTTCGTTCAACTTTATGGT
P K L L T F D L T T S Q L L K Q V E I P
133 135 137 139 141 143 145 147 149 151
CATGATGTTGCCGTAAATGCCACTACAGGAAAGGGAAGATTATCATCTCTAGCTGTTCAA
GTACTACAACGGCATTTACGGTGATGTCCTTTCCCTTCTAATAGTAGAGATCGACAAGTT
H D V A V N A T T G K G R L S S L A V Q
153 155 157 159 161 163 165 167 169 171
TCTTTAGATTGCAATACAAATAGCGATACTATGGTGTATATAGCAGACGAGAAAGGAGAA
AGAAATCTAACGTTATGTTTATCGCTATGATACCACATATATCGTCTGCTCTTTCCTCTT
S L D C N T N S D T M V Y I A D E K G E
173 175 177 179 181 183 185 187 189 191
ClaI
GGTTTAATCGTGTATCATAATTCTGATGATTCTTTCCATCGATTGACTTCCAACACTTTC
CCAAATTAGCACATAGTATTAAGACTACTAAGAAAGGTAGCTAACTGAAGGTTGTGAAAG
G L I V Y H N S D D S F H R L T S N T F
193 195 197 199 201 203 205 207 209 211
GATTACGATCCTAAATTTACCAAAATGACCATTGATGGAGAAAGTTACACAGCCCAAGAT
CTAATGCTAGGATTTAAATGGTTTTACTGGTAACTACCTCTTTCAATGTGTCGGGTTCTA
D Y D P K F T K M T I D G E S Y T A Q D
213 215 217 219 221 223 225 227 229 231
GGAATTTCTGGAATGGCTCTTAGTCCCATGACTAACAATCTCTATTACAGTCCTGTAGCT
CCTTAAAGACCTTACCGAGAATCAGGGTACTGATTGTTAGAGATAATGTCAGGACATCGA
G I S G M A L S P M T N N L Y Y S P V A
233 235 237 239 241 243 245 247 249 251
HincII
TCCACCAGTTTGTATTATGTTAACACGGAACAATTCAGAACATCCGATTATCAACAGAAT

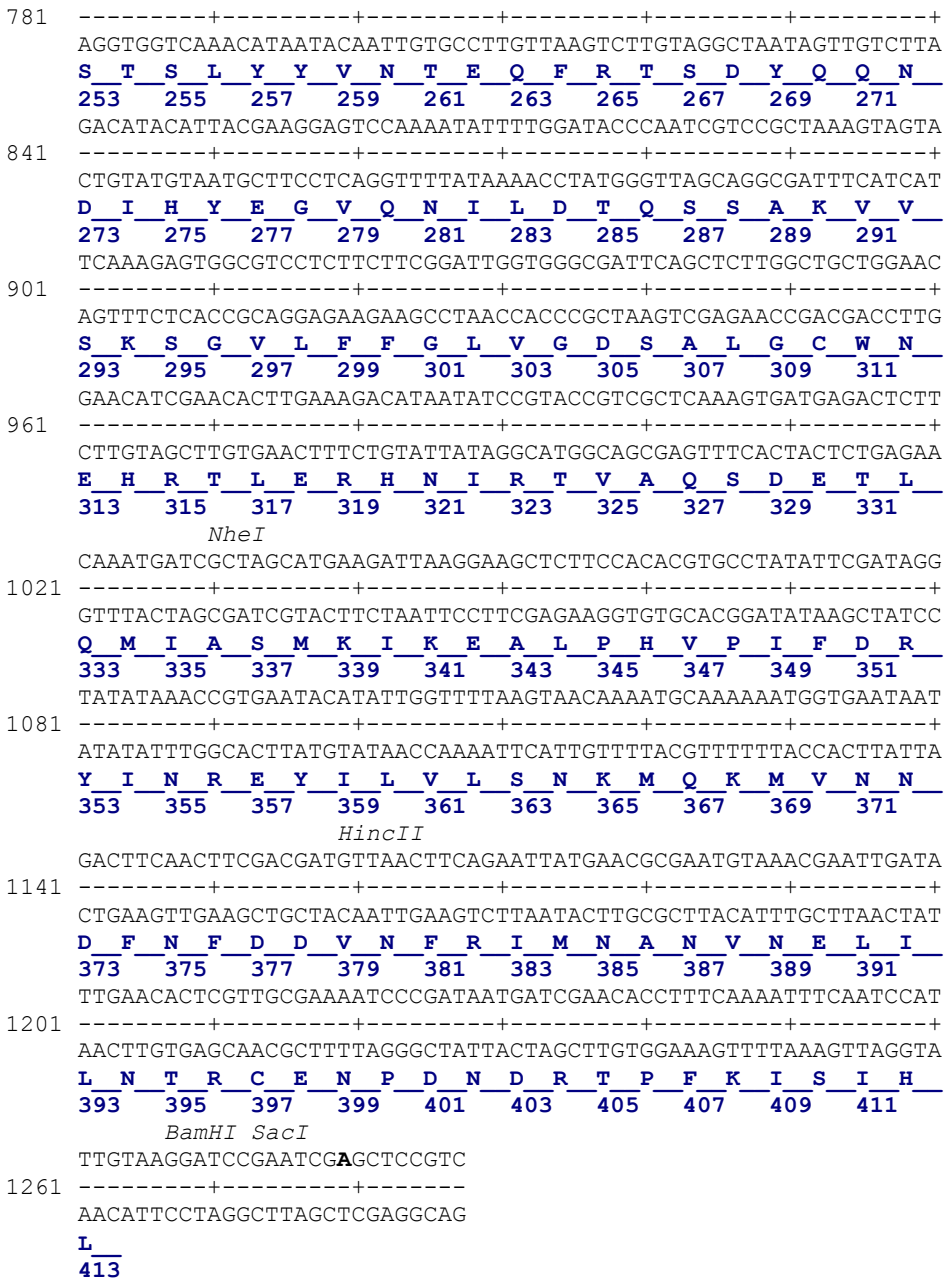



*Figure 3.2 Royalactin cDNA and relative protein sequence. Taken from GenBank (accession number GQ160518.1).*

### 3.2.2 Transformation and expression using different *E. coli* strains

All *E. coli* strains that were used are engineered to express the recombinant protein after being induced by IPTG. All the samples obtained from the expressions were run in SDS-PAGE gels prepared by me following the protocol in section 2.2.15, and the

electrophoresis was run following the protocol in section 2.2.16. The basic expression protocol was obtained from Kamakura (Kamakura, 2011). The protocol (section 2.2.5) was not amended except for the use of a different plasmid: pET28b.

All the *E. coli* strains were transformed with the pET28b vector containing Royalactin's cDNA, named pET28:RA, using the protocol described in section 2.2.4. A glycerol stock (section 2.2.7) of each transformed strain was created for long term storage in the -80°C freezer. LB agar plates containing the appropriate antibiotics (section 2.2.2) were created every two weeks and kept in the fridge to maintain the strain fresh for the experiments.

50 mL of room-temperature LB medium were put in a 250 mL flask to which the appropriate amount of antibiotics was added (section 2.1.10). The flask was then inoculated with a single colony from a previously streaked LB agar plate containing the appropriate antibiotics, the flask was then incubated overnight in a shaker at the strain's appropriate temperature. The next day, 25 mL of the bacterial culture were used to inoculate 500 mL of LB medium with no antibiotics added. The flask was incubated in a shaker at the appropriate temperature for around 2 hours, then 750 µL of culture were taken to measure the absorbance using a spectrophotometer. When the absorbance ($O.D._{600}$) reached a value between 0.4 and 0.8, the culture was induced by adding IPTG with a final concentration of 1 mM.

#### 3.2.2.1 BL21 (DE3)

*Escherichia coli* BL21 (DE3) cells are deficient in Lon protease, a cytoplasmic protein, that degrades misfolded proteins, and OmpT protease, an outer membrane protein,

that helps the cell to collect free amino acids from the environment (https://international.neb.com/tools-and-resources/selection-charts/competent-cell-selection-guide). Without those two proteases the *Escherichia coli* strain is claimed to have more manageable expression for the production of recombinant proteins. DE3 indicates that the strain contains the λDE3 lysogen (cells carry a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter). This modification makes the strain suitable for protein production by induction with IPTG.

All experiments followed the protocol described above, but since no expression was achieved, temperature and IPTG concentration were modified. Expression time was also modified to test expression between 4 and 24 hours after the induction. No expression was attained using this strain. Protocol changes are listed in Table 3.3.

#### 3.2.2.2 Origami 2 (DE3) *pLysS*

Origami 2 was chosen as the second strain because it has mutations that enhance the folding of the expressed protein. This strain was chosen because the lack of expression using BL21 (DE3) could be due to BL21's inability of properly folding the recombinant protein (https://international.neb.com/tools-and-resources/selection-charts/strain-properties). The manufacturer's expression protocol was used, but in addition to that, I tested the theory that the protein could be produced and immediately stored in inclusion bodies (clusters of heavy packed protein that make it insoluble). Since the SDS-PAGE samples are prepared using the supernatant of whole cell lysate, it could have been possible that the insoluble proteins would not have been visible. To break the

inclusion bodies, I used centrifugation alternating with salt washes to solubilise the protein to be able to run it on an SDS-PAGE gel.

All experiments followed the protocol described above. As with the strain above, no expression was achieved and temperature and IPTG concentration were modified. Expression time was also modified to test expression between 17 and 23 hours after the induction. No expression was attained using this strain. Protocol changes are listed in Table 3.4.

#### 3.2.2.3 Lemo 21 (DE3)

Lemo 21 is a strain engineered to express toxic proteins and proteins likely to insoluble expression. This strain was chosen after failures with the other two, and because some sources classify Royalactin as having some antibiotic properties (Fontana et al., 2004). Lemo 21 has tuneable expression, using L-rhamnose, which concentration can vary between 0 and 2000 µM, all the experiments were performed using 100 µM following the manufacturer's guidelines that show that this concentration is the most effective in expressing the membrane protein PhoA. The use of Lemo 21 proved successful and I was able to observe a band forming and becoming stronger with time after the induction. The band was excised from the SDS-PAGE gel and was sent for sequencing at the University of York, Department of Biology, Metabolomics and Proteomics Laboratory: this confirmed that it is Royalactin.

IPTG concentration was modified to try to obtain a higher yield. The initial culture O.D. value was investigated as well, to understand how the density of cells in the initial cultivation affects production.

#### 3.2.2.4 SHuffle T7 Express *lysY*

SHuffle was the fourth strain that I used; it is engineered to promote disulphide bond formation in the cytoplasm, thanks to a disulphide bond isomerase that also acts as a chaperone to promote correct folding of the protein, independent of disulphide bond formation. Being a *lysY* strain means that the cell line expresses lysozyme to better control basal protein expression, making it possible to produce toxic proteins. SHuffle grows at 30° C which assists with slower protein expression and a resulting lower probability of insolubility (https://international.neb.com/tools-and-resources/selection-charts/strain-properties).

Figure 3.9 shows the expression of a band in both SHuffle and Lemo strains, with the strongest band for Lemo at T22. SHuffle was not used again, since Lemo had a better expression.

### 3.2.3 *In vitro* expression

Before experimenting with *E. coli* strain Lemo 21, I decided to try to produce Royalactin through *in vitro* expression. Two different transcription systems from two companies were used: mMessage mMachine (Thermo Fisher Scientifics) and Riboprobe *in vitro* transcription system (Promega), following the manufacturer's protocol (section 2.2.20). For the translation Rabbit Reticulocyte lysate (Promega) was used, following the manufacturer's protocol (section 2.2.21). Figure 3.3 is a schematic representation of the process of the *in vitro* expression.

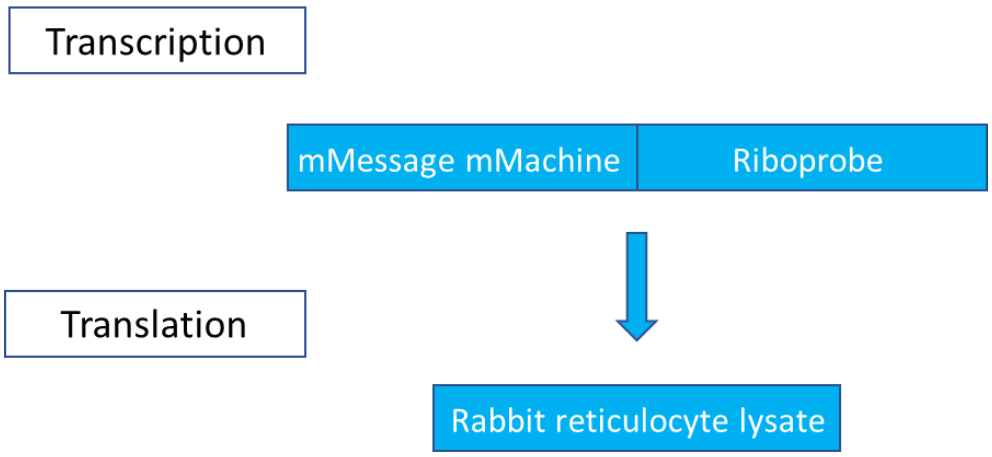



*Figure 3.3 In vitro expression schematisation.*

### 3.2.4 Protein purification

The purification efforts were all based on the same principle of affinity chromatography, which is a method of separating a target molecule through a specific interaction between a receptor and the ligand (the HIS-tag). All products used are based on nickel affinity, in which immobilised metal ions bind to the HIS-tag linked to the protein. The bond is cleaved using imidazole, which changes the conditions of the buffer, the affinity chromatography can then be used again. Three different products were used: Nickel-NTA Magnetic Agarose Resin (5%) (ABT), Nickel Affinity Cartridge (5ml) (ABT), Capturem His-Tagged Purification Miniprep Kit (TAKARA). The nickel affinity cartridge was also used paired with the ÄKTA explorer chromatography system (GE Healthcare) (Table 3.2).

The nickel-NTA magnetic agarose resin (5%) was used following the protocol described in section 2.2.18.3. This resin consists of magnetic agarose derivatized with Nitrilotriacetic (NTA), it is composed of resin coated magnetic beads that can be collected using a magnet to allow an easy separation of the target protein from the rest of the sample.

The Capturem His-Tagged Purification Miniprep Kit was used following the protocol described in section 2.2.18.1. This system is based on the use of nylon-based membranes to capture the target protein, the solution is loaded into spin columns that can be used with a tabletop centrifuge.

The Nickel NTA Agarose Cartridge was used following the protocol described in section 2.2.18.2. The column is pre-packed with a Nickel NTA agarose resin, working on the exact same resin as the magnetic beads. The column can be used either paired to a chromatography system or by using a syringe to deliver the sample and the washes.

*Table 3.2 Purification techniques used on E. coli Lemo21 (DE3) cells.*

| Lemo 21 | Conditions | Outcome |
|---|---|---|
| Magnetic Beads | Natural | Negative |
| Capturem | Natural | Negative |
| Nickel Affinity Cartridge | Natural | Negative |
| Nickel Affinity Cartridge using ÄKTAexplorer chromatography system | Natural & denaturing | Negative |

For the first three methods, the manufacturer's protocols were followed; these involved natural conditions that keep the protein's tertiary structure intact. Conversely, denaturing conditions remove these tertiary structures due to the use of chaotropic agent, such as urea, for promoting solubilization. ABT's nickel affinity cartridge was used both manually and in the chromatography system ÄKTAexplorer to automate the purification process; both natural and denaturing conditions were used with the ÄKTA system. A gradient of imidazole concentration in the elution buffer was employed, with concentrations varying between 10 mM and 2 M, to study how and when the protein

disassociates from the column. Denaturing conditions were also used in the washing and elution buffers, which also contain 8 M urea.

### 3.2.5 Protein identification

Identification was outsourced to University of York, Department of Biology, Metabolomics and Proteomics Laboratory. Protein identification is performed by Matrix Assisted Laser Desorption Ionization Tandem Time-of-Flight mass spectrometry (MALDI TOF M/S). The protein is digested with trypsin and then analysed with MALDI TOF M/S; the ten strongest peaks are selected for MS/MS fragmentation. The data generated is searched against the UniProt database using Mascot (http://www.matrixscience.com/help.html), which gives expected values associated with every peptide. This method generates peptide matches that are used to infer protein identification. Usually an identification is successful when a minimum of two peptides are matched to a protein below a justifiable threshold (Cottrell, 2011). The threshold is the ratio of false positives per search, which is the number of times a random peptide is matched. The default threshold of the program is 0.05, which is 1 false positive in 20 searches; only matches with an expected value lower than 0.05 are shown.

## 3.3 Results

### 3.3.1 Production of Royalactin using *E. coli* cells

#### 3.3.1.1 BL21 (DE3)

Efforts to express Royalactin using *E. coli* BL21 (DE3) cells or by using Kamakura's protocol did not produce any band in the correct and expected value of molecular weight (47kDa), using SDS-PAGE gels (section 2.2.12).

The list of experiments that were performed using BL21 (DE3) is given in Table 3.3.

*Table 3.3 Expression of Royalactin using E. coli BL21 (DE3) cells.*

| BL21 (DE3) | Temperature | Time | IPTG concentration | Outcome |
|---|---|---|---|---|
| Expression | 37° C | 24 h | 1 mM | Negative |
| Expression | 30° C | 22 h | 0.5 mM | Negative |
| Expression | 30° C | 22 h | 1 mM | Negative |
| Expression | 30° C | 4 h | 2 mM | Negative |
| Expression | 30° C | 4 h | 4 mM | Negative |
| Expression | 37° C | 4 h | 1 mM | Negative |
| Expression | 37° C | 4 h | 2 mM | Negative |
| Expression | 37° C | 4 h | 4 mM | Negative |

None of the experiments above produced a clear 47kDa band when the whole cell lysate was run in an SDS-PAGE gel.

Figure 3.4 shows an example of expression using BL21 (DE3) and then running its whole cell lysate in an SDS-PAGE gel. In this example, the cells were induced with 1mM IPTG and grown at 37° C. Samples were taken on induction and then 7, 10, 14, 15, 17, 19, 20 and 24 hours after. A positive result would have shown a band of 47 kDa growing stronger with time.

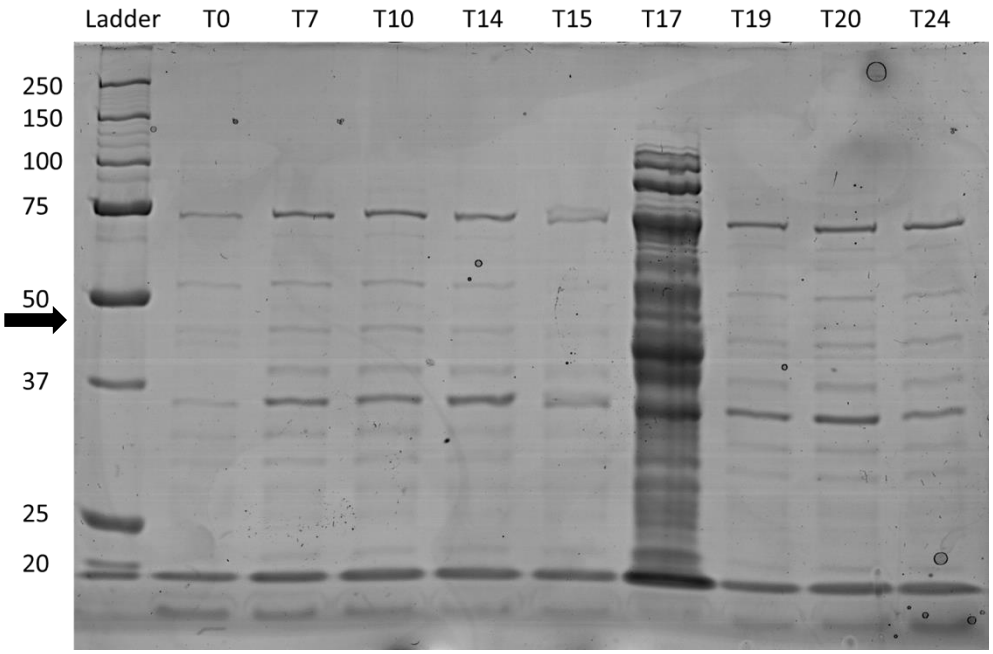



*Figure 3.4 SDS-PAGE gel of induction of BL21 (DE3) with IPTG 1mM, supernatant of whole cell lysate. Sampled on induction, 7, 10, 14, 15, 17, 19, 20 and 24 hours after. 10µL of sample were loaded in the gel for each sample to normalise for volume. The black arrow indicates the expected molecular weight of Royalactin. Ladder is BIO-RAD Dual Colour.*

To ensure that the technique was correct, a plasmid containing the bacterial luciferase protein (LuxA) was transformed into the same strain of BL21 (DE3) *E. coli* as a form of positive control. It was then incubated using the same parameters alongside the bacteria containing Royalactin. LuxA dimension is 40 kDa, expression using BL21 (DE3) containing the plasmid for LuxA was successful as can be seen in Figure 3.5.

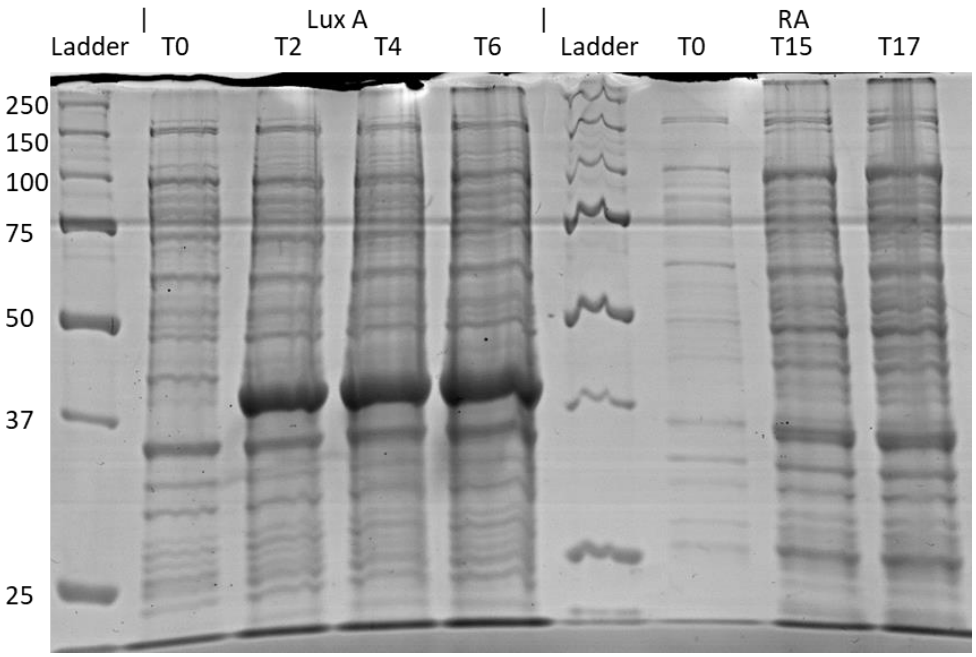



*Figure 3.5 SDS-PAGE gel of comparison of whole cell lysate for two BL21 (DE3) cultures with plasmids containing two different inserts: LuxA and Royalactin. Induced with IPTG 1 mM. Grown at 37 °C and sampled on induction, 2h, 4h and 6h for LuxA, and induction, T15 and T17 for Royalactin. 10µL of sample were loaded in the gel for each sample to normalise for volume. Ladder is BIO-RAD Dual Colour.*

### *3.3.1.2* Origami 2 (DE3) *pLysS*

Table 3.4 lists the experiments performed using *E. coli* cell line Origami 2. Expression with Origami 2 was unsuccessful, as it was using the inclusion bodies technique.

*Table 3.4 Expression of Royalactin using E. coli Origami2 (DE3) pLysS cells.*

| Origami 2 | Temperature | Time | IPTG concentration | Outcome |
|---|---|---|---|---|
| Expression | 37° C | 23 h | 1 mM | Negative |
| Inclusion bodies | 37° C | 17 h | 1 mM | Negative |
| Whole cell lysate | 37° C | 17 h | 1 mM | Negative |

Figure 3.6 shows the comparison between the whole cell lysate of Origami 2 and the same samples treated with the inclusion bodies technique. Cells were induced with 1mM IPTG and grown at 37° C. Samples were taken on induction and then 15 and 17 hours after. A band of 47 kDa grow can be seen, but it has the same size of a band already present at induction, which suggests that it was a pre-existing band that has grown stronger due to the higher number of cells present after being incubated for 15 and 17 hours.

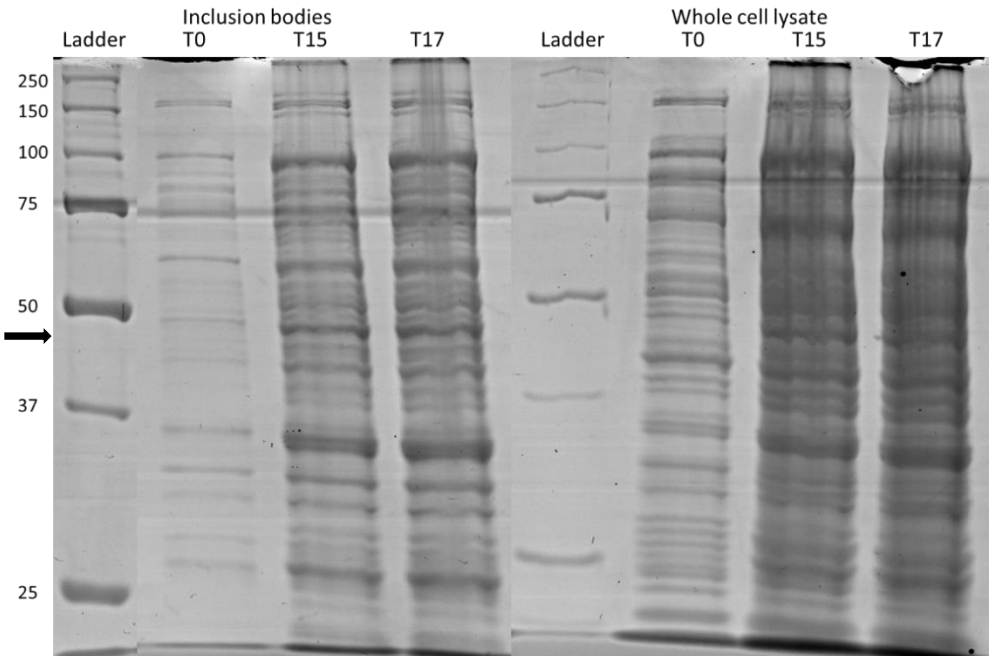



*Figure 3.6 SDS-PAGE gel of induction of Origami 2 with IPTG 1mM, grown at 37° C. In the first four (left) columns we used the inclusion bodies technique. In the last four columns (right) we used the standard protocol. Sampled on induction, 15, and 17 hours after. The black arrow indicates the expected molecular weight of Royalactin. Ladder is BIO-RAD Dual Colour.*

As done previously with the BL21 (DE3) strain, a positive control was set up transforming a plasmid containing the bacterial luciferase protein LuxA into BL21. Success in expressing this protein means that the procedure works. In Figure 3.7 the LuxA sample was collected after 4 hours and its gel bands were compared to previously obtained LuxA gel bands to assess the strength of the expression.

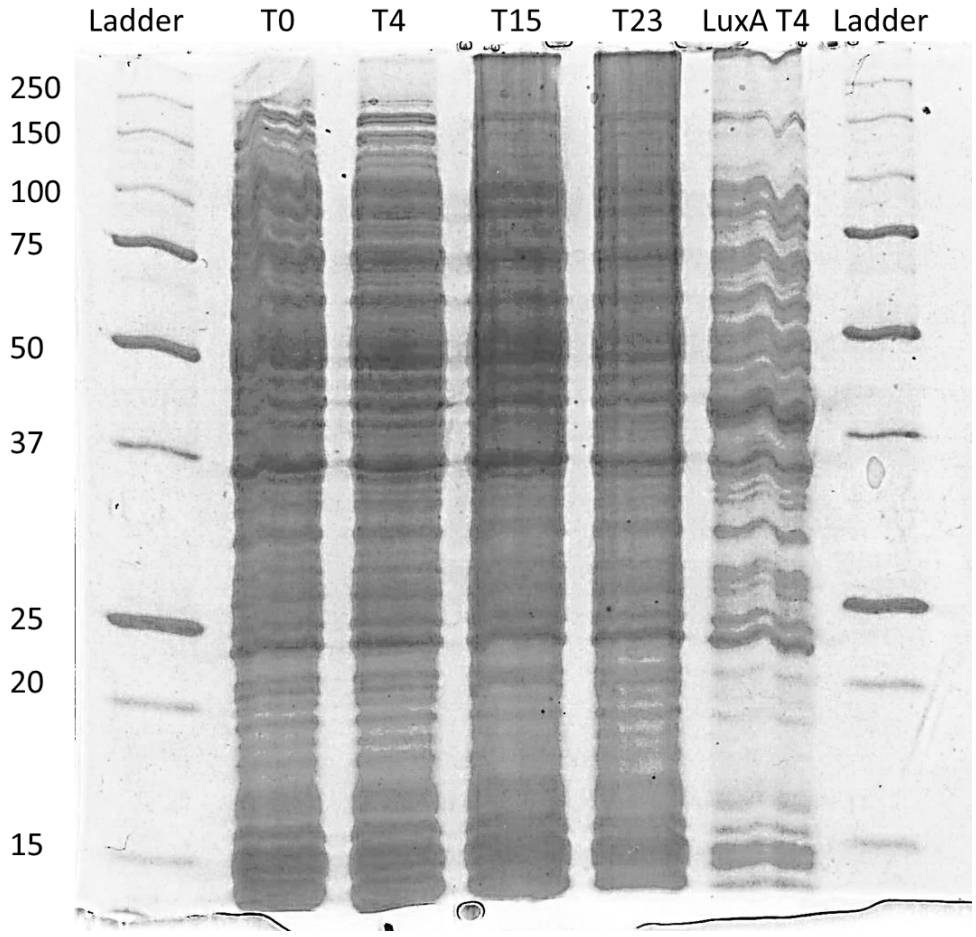



*Figure 3.7 SDS-PAGE gel of comparison of whole cell lysate for two Origami 2 cultures with plasmids containing two different inserts: LuxA and Royalactin. Induced with IPTG 1 mM. Grown at 37 °C and sampled on induction, 4h, 15h and 24h for Royalactin, and induction and T4 for LuxA. 10µL of sample were loaded in the gel for each sample to normalise for volume. Ladder is BIO-RAD Dual Colour.*

#### 3.3.1.3 Lemo 21 (DE3)

Figure 3.8 shows the comparison of the protein bands between Lemo21 expressing Royalactin (pET28:RA) versus Lemo21 not containing any vector. Both were sampled on induction and 17 hours after. A strong band can be seen on the Royalactin expressing Lemo21 sample after 17 hours of incubation. The same expression samples were run in

a western blot as can be seen in Figure 3.8. Mouse monoclonal antibody to 6XHis-tag (ABCAM), and Goat polyclonal antibody to mouse IgG (HRP) (ABCAM) were used to highlight the presence of a strong band around 47 kDa on the sample of Lemo21 expressing Royalactin that was induced for 17 hours. The antibodies' dilution is listed in Table 2.2, following the technique described in 2.2.19.

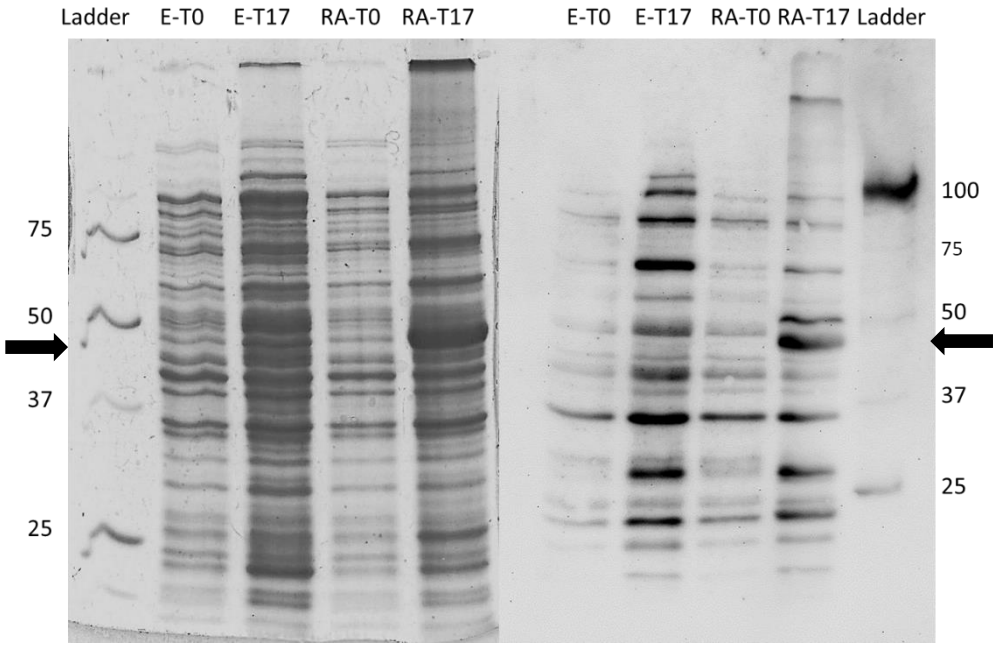



*Figure 3.8 Comparison between western blot and induction of Lemo21 pET28 and Lemo21 pET28:RA, induced with IPTG 1mM. Samples were sampled on induction and after17 hours. Samples are marked with a capital E for Lemo21 transformed with an empty vector, and with a capital RA for Lemo21 transformed with a plasmid containing Royalactin. 10µL of sample were loaded in the gel for each sample to normalise for volume. The black arrows indicate the expected molecular weight of Royalactin. Ladder for SDS-PAGE is BIO-RAD Dual Colour and ladder for western blot is Amersham ECL DualVue Western blotting markers.*

A positive control was added to assess the expression's success by transforming a plasmid containing the bacterial luciferase protein LuxA into BL21 and then incubating and expressing both strains at the same time (Figure 3.9).

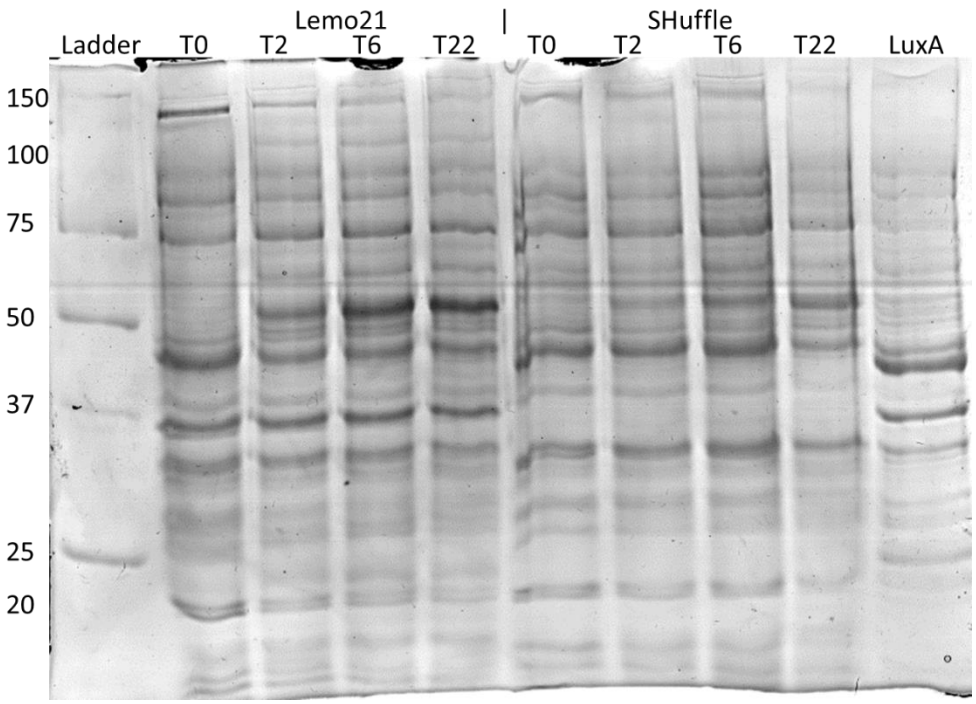



*Figure 3.9 Comparison of induction of Lemo21 strain and SHuffle, induced with IPTG 1mM. Grown at 37° C. Sampled on induction, 2h, 6h and 22h. LuxA was sampled at 4h. 10µL of sample were loaded in the gel for each sample to normalise for volume. Ladder is BIO-RAD Dual Colour.*

To improve the yield, IPTG concentration was tested between 0.5 and 1.75 mM. Using 1.25 mM IPTG gave the best yield, as can be seen in Figure 3.10.

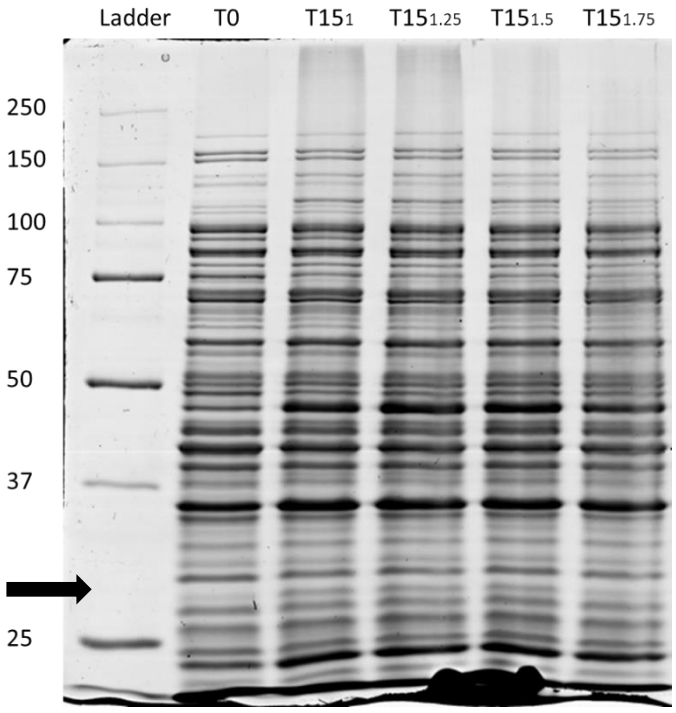



*Figure 3.10 Comparison of four different inductions of Lemo21 with different IPTG concentrations: 1 mM, 1.25 mM, 1.5 mM and 1.75 mM. Sampled on induction and 15h. 10µL of sample were loaded in the gel for each sample to normalise for volume. The black arrow indicates the expected molecular weight of Royalactin. Ladder is BIO-RAD Dual Colour.*

#### *3.3.1.4* SHuffle T7 Express *lysY*

Figure 3.9 shows the comparison between the expression of Lemo21 and SHuffle. Both strains were sampled on induction 2, 6 and 22 hours after. The emergence of a band around 47 kDa can be seen in both strains, with the production being greater in Lemo21. Royalactin's production was achieved with both strains; no quantitative analysis was performed but a visual qualitative analysis using Lemo21 showed stronger bands compared to SHuffle.

### 3.3.2 *In vitro* expression

The expected result of the *in vitro* transcription was an RNA band of around 1.3 kilobases long, but neither the mMessage mMachine kit nor the Riboprobe kit produced any bands (Figure 3.11). Samples of the transcription were run through translation, but no protein band of 47 kDa was visible either (not shown). Rabbit reticulocyte lysate was used pure to evaluate its protein pattern.

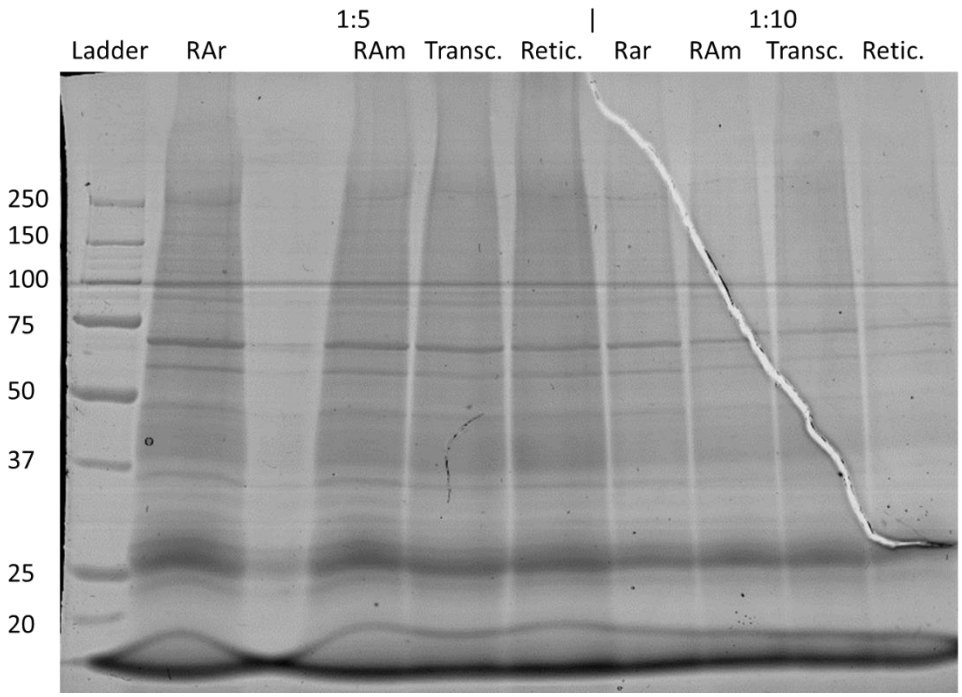



*Figure 3.11 Comparison of in vitro transcription kits in dilutions of 1 to 5 and 1 to 10. mMessage mMachine T7 transcription kit (Thermo Fisher Scientific) is marked as RAm, and Riboprobe In Vitro Transcription Systems kit (Promega) is marked as RAr. 10µL of sample were loaded in the gel for each sample to normalise for volume. Ladder is BIO-RAD Dual Colour.*

A critical mistake was found after having performed the experiment where leucine was not added to the *in vitro* translation mix. This is likely the reason the experiment was unsuccessful.

### 3.3.3 Protein identification

To confirm the identity of Royalactin, Lemo21 pET28b:RA was incubated and sampled after induction and after 23 hours. The samples were sonicated to open all bacterial cells and centrifuged to remove cell membranes and DNA. A 12% SDS-PAGE gel was used instead of the usual 10% concentration, to increase the resolution of proteins with

molecular weights in the range of Royalactin's expected size, this allowed for a more precise excision of individual protein bands. Special care was taken during the preparation of the gel to avoid keratin contamination. Keratin contamination is caused by user manipulation, keratin will hide any other target protein during mass spectrometry because of its abundance. The induction and 23 hours incubation samples were run in an SDS-PAGE gel and the 47 kDa bands were cut and sent to identification to the Department of Biology, Metabolomics and Proteomics Laboratory of the University of York, where the peptides were searched against UniProt database and a University of York in-house database.

#### 3.3.3.1 Induction sample

Time 0 band's peptides were searched against UniProt database to obtain information on the band that Royalactin overlaps after being expressed. The match is *E. coli*'s (strain K12) ATP synthase subunit beta. This is in line with the bands belonging to an *E. coli* that has not started expressing any proteins. The values in the "Expected value" column should be lower than the threshold of 0.05 for the peptide to be considered a valid match with the protein. The threshold represents a one in 20 chance to produce a false positive.

*Table 3.5 Induction sample band against the UniProt database.*

| **Peptide name** | **Expected value** |
|---|---|
| R.DVLLFVDNIYR.Y | $5.9^{-6}$ |
| R.YTLAGTEVSALLGR.M | $1.2^{-10}$ |
| R.VYDALEVQNGNER.L | 0.021 |
| R.LVLEVQQQLGGGIVR.T | $6.6^{-6}$ |
| K.VSLVYGQMNEPPGNR.L | 0.019 |
| R.QLDPLVVGQEHYDTAR.G | $1.5^{-6}$ |
| R.NIAIEHSGYSVFAGVGER.T | $3.4^{-10}$ |

#### 3.3.3.2 23 hours incubation sample

Mass spectrometry analysis produced peptide sequences that were searched against UniProt database producing 3 hits against MRJP1, while when searched against the in-house database, they produced 4 matches against the Royalactin's sequence that was provided by me, which was the same as used by Kamakura (Kamakura, 2011).

Table 3.6 and Table 3.7 show on the far-left column the name of the peptides that matched in the UniProt and the University of York's in-house databases. The values of the "Expect" column should be lower than the threshold of 0.05 to consider the peptide a valid match with the protein.

*Table 3.6 23 hours incubation sample band against the UniProt database.*

| Peptide | Expected value |
|---|---|
| K.EALPHVPIFDR.Y | 0.0006 |
| K.FFDYDFGSDER.R | $6.9^{-5}$ |
| R.IMNANVNELILNTR.C | $4.2^{-6}$ |

*Table 3.7 23 hours incubation sample band against the in-house database.*

| Peptide | Expected value |
|---|---|
| K.FFDYDFGSDER.R | $1.7^{-7}$ |
| K.IFVTMLR.Y | 0.028 |
| K.EALPHVPIFDR.Y | $8^{-7}$ |
| R.IMNANVNELILNTR.C | $5.5^{-9}$ |

These seven matches and their expected values confirm that the protein expressed by inducing Lemo21 containing the Royalactin plasmid, was indeed Royalactin.

### 3.3.4 Protein purification

Purification efforts were unsuccessful because the protein was being washed away before the elution step, as can be seen in Figure 3.12 and Figure 3.13. When comparing

the whole-cell-lysate sample to the first flow-through, the band profiles are identical (Figure 3.12). The protein did not seem to attach to the column and could be washed away, even with very small amount of imidazole, with the rest of the non-target proteins (Figure 3.13 and Figure 3.14).

Figure 3.12 shows the comparison between the bands of Lemo21 pre-induction (T0), the bands of Lemo21 19 hours after induction including a strong Royalactin band, and finally the bands after using the Nickel Affinity Cartridge (ABT) (section 2.2.18.2). The Royalactin band is not visible in the purified sample.

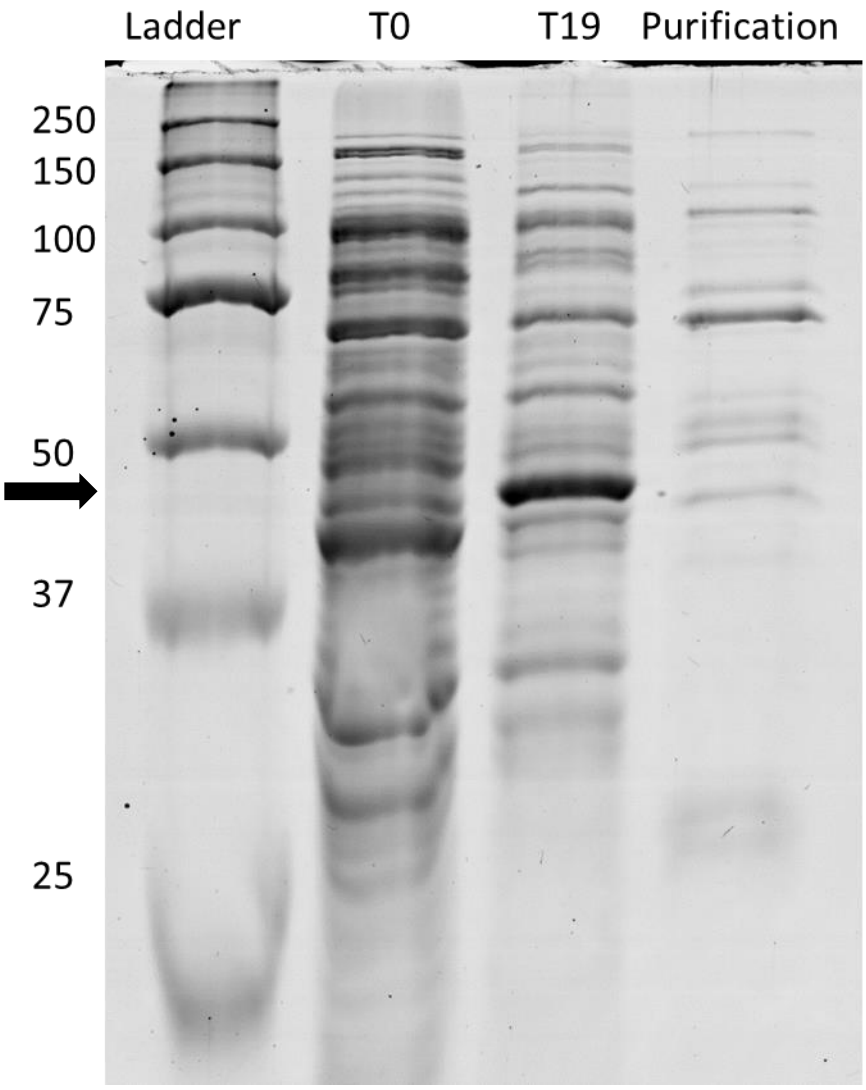



*Figure 3.12 Purification of T19 Lemo21 induced with IPTG 1.25 mM. Columns T0 and T19 represent whole cell lysate sample at induction and after 19 hours of expression, respectively. Purification performed using Nickel Affinity Cartridge (ABT). 10µL of sample were loaded in the gel for each sample to normalise for volume. The black arrow indicates the expected molecular weight of Royalactin. Ladder is BIO-RAD Dual Colour.*

Figure 3.13 shows the comparison between the bands of Lemo21 pre-induction (T0), the bands of Lemo21 15 hours after induction using 1.75mM IPTG ($T15_{1.75}$), and finally the bands after using the Capturem His-Tagged Purification Miniprep Kit (Takara)

(section 2.2.18.1). The Royalactin band is not visible either in the washes or in the purified sample.

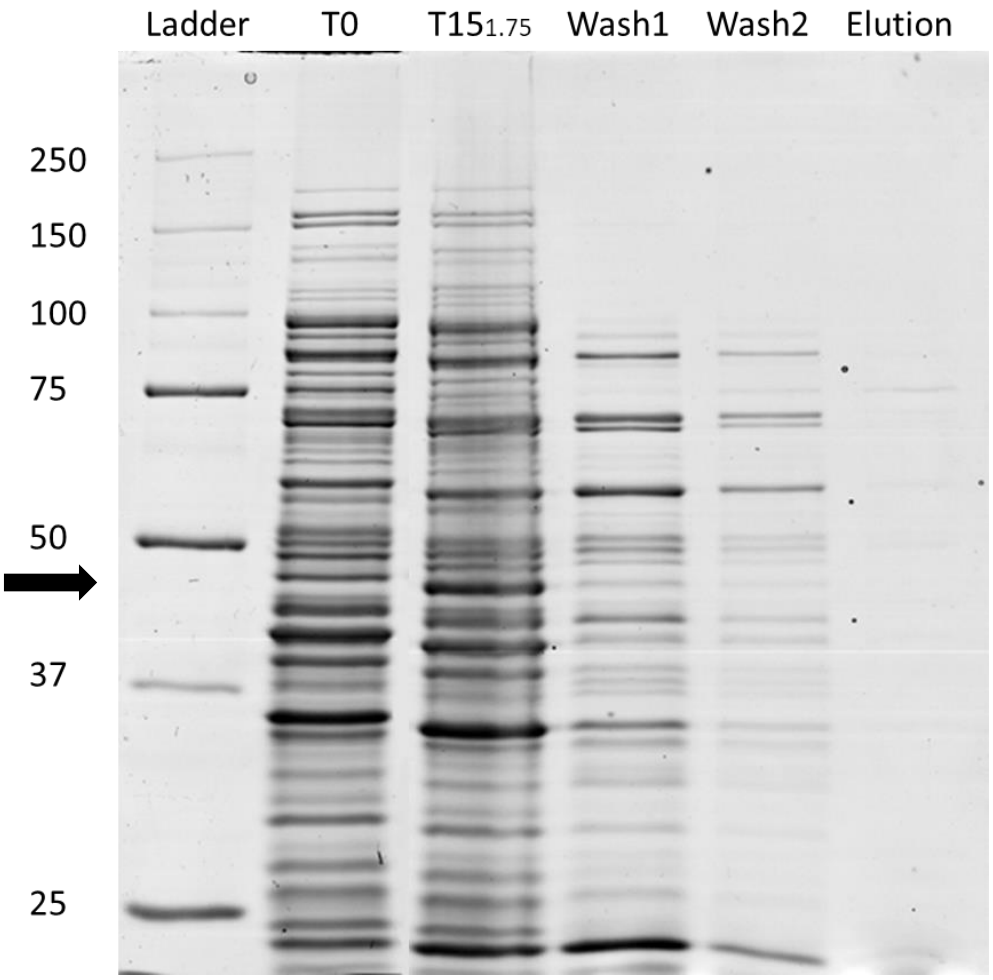



*Figure 3.13 Purification of Lemo21 T15 induced with 1.75 mM IPTG. The gel includes expression samples of uninduced whole cell lysate, whole cell lysate after 15 hours, and purification samples of the two washes and the elution step. Purified using Capturem His-Tagged Purification Miniprep Kit (Takara). 10µL of sample were loaded in the gel for each sample to normalise for volume. The black arrow indicates the expected molecular weight of Royalactin. Ladder is BIO-RAD Dual Colour.*

Figure 3.14 shows the attempted purification using a Nickel Affinity Cartridge (ABT) with ÄKTA chromatography system in denaturing conditions. Shown are the bands of Lemo21 before induction (T0), the bands of Lemo21 19 hours after induction (T19), the flow through of the sample through the column, and finally the bands of the washes

with a gradient of imidazole from 10mM to 1M. The Royalactin band is not visible either in the washes or in the purified sample.

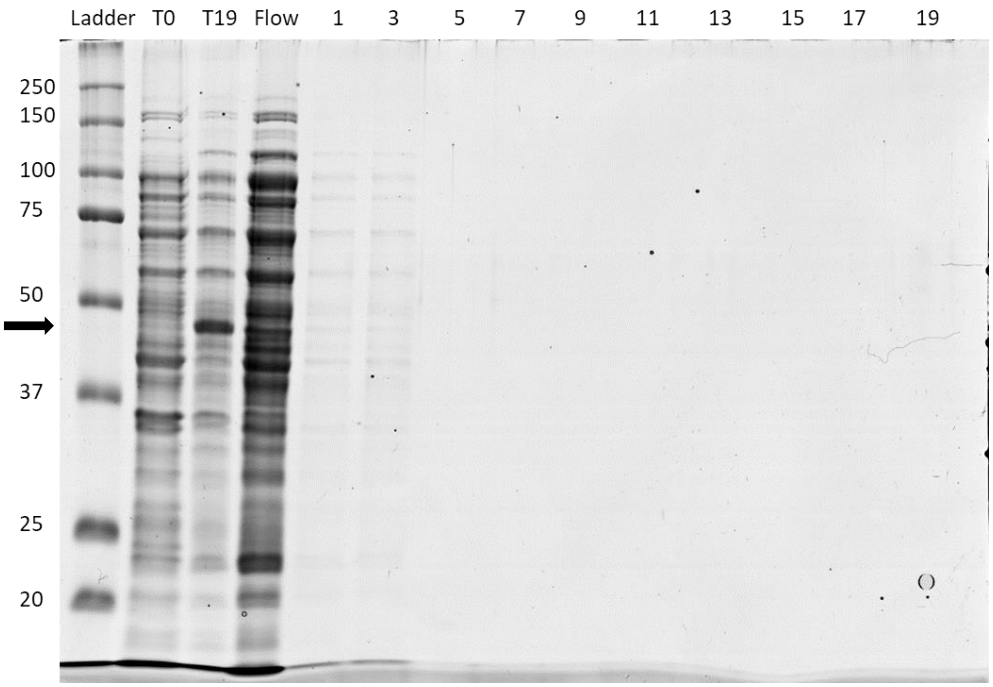



*Figure 3.14 Purification of T19 Lemo21 using a Nickel Affinity Cartridge (ABT) with ÄKTA chromatography system in denaturing conditions. The gel includes expression samples of uninduced whole cell lysate, whole cell lysate after 19 hours, and purification samples of the flowthrough and the elution steps. 10µL of sample were loaded in the gel for each sample to normalise for volume. The black arrow indicates the expected molecular weight of Royalactin. Induced with IPTG 1.25 mM. Ladder is BIO-RAD Dual Colour.*

## 3.4 Discussion

In this chapter, I investigated the expression of Royalactin using bacterial cell lines and its purification from the bacterial proteins. I followed the protocol used by Kamakura

(Kamakura, 2011) but obtained no expression. I then was able to express the protein by changing the bacterial strain to one able to produce toxic proteins. I demonstrated how the protein is not found in the insoluble part of the whole cell lysate and how the modification of IPTG concentration influences the final expression.

### 3.4.1 Royalactin expression

Expressing Royalactin was more difficult than expected: using Kamakura's protocol did not lead to positive results as four different bacterial strains had to be tried to obtain expression. Using a strain capable of expressing toxic proteins (both Lemo and Shuffle) made it possible to produce Royalactin and it was confirmed by sequencing the protein. The initial inability to produce Royalactin could be attributable to the toxicity of the protein (Fontana et al., 2004); the only strains that were able to express it had in common an ability to reduce basal expression of T7 polymerase, which is involved in the production of toxic proteins. Origami 2 also has the *pLysS* plasmid but this did not help with the expression of Royalactin, possibly due to not having reached the strain's correct expression conditions.

#### 3.4.1.1 Alternative expression systems

There are other systems that allow the production of recombinant proteins and some of them have been used for the expression of Royalactin. Judova et al. (1998) transformed the cDNA of major royal jelly protein into tobacco plants to enhance the plants nutrients. Although they achieved expression, its level was not satisfactory to be used as a supplement of Royalactin, which was what the group had planned. This is still a viable form of expression, since plants possess the ability to perform post-translation

modifications, such as glycosylating the expressed proteins, something that is not possible using bacteria.

The yeast *Pichia pastoris* is another organism used to produce recombinant Royalactin, with the same intentions of improving on the use of bacteria expression. Shen et al. (2010) argue that the use of *P. pastoris* has many benefits over the use of bacteria: it avoids the risk of producing an insoluble protein or a protein that creates inclusion bodies, it allows for glycosylation and postprocessing and, therefore, the lack of biological activity. Ibarra-Herrera et al. (2014) used *P. pastoris* with the intent of overcoming the same problems that occur using bacteria or plants, specifically the formation of inclusion bodies and the low levels of biological activity using *E. coli*, and the low expression levels presented in tobacco leaf.

*P. pastoris* possesses the capability of performing varying types of post-translational modifications, such as disulphide bond formation, glycosylation, and eukaryotic proteolytic processing, it is also able to secrete heterologous eukaryotic proteins in their native, biologically functional form (Cereghino and Cregg, 2000). This also comes with a more difficult purification part compared to bacterial purification, as stated in Ibarra-Herrera et al. (2014), but the benefits outweigh the costs.

### 3.4.2 Optimisation of growth conditions

The optimisation experiments performed on the strains targeted growth temperature, O.D. and IPTG concentration. The results show that neither temperature nor initial O.D. changed the outcome when using BL21 and Origami 2 strains. The modification of IPTG concentration in Lemo, on the other hand, produces a curve of expression, with the

peak reached between concentrations of 1.25 and 1.5 mM. 1.25 mM became the standard concentration. Initial O.D. does not change the outcome in Lemo.

### 3.4.3 Protein purification

The purification efforts were unsuccessful, the protein did not bind to the column and was washed away with the rest of the proteins. This could be caused by the quaternary structure of the protein, which could hide the HIS-tag. The free assembly into a four-branched structure was reported by Šimúth (2001) using Royalactin obtained through ultracentrifugation. The formation of oligomers from monomers would lead to large proteins with the possibility of hiding the HIS-tag or aggregates that are too large to penetrate the pores of the gel. This spontaneous arrangement into an oligomer possibly confers enhanced stability compared to the monomeric form (Eisenstein and Schachman, 1989; Tamura et al., 2009) but could have the side-effect of reducing the protein's capacity of linking to the purification matrix.

Denaturing conditions were used to remove the protein's tertiary and quaternary structures, but the protein did not bind to the column either. A follow up to the expression experiments would be to prepare a western blot against the His-tag and test its presence in all steps of the purification: from the flowthrough to the washes and finally to the elution step. This would give a clear image of where the protein goes missing, allowing for better troubleshooting.

## 3.5 Conclusions

The initial aim of producing recombinant Royalactin, purifying it and then feeding it to insects was hindered by the difficulty to properly purify the protein and remove it from

*E. coli*'s proteins. Using an un-purified protein would have added a plethora of other nutritional sources to the diet, defeating the purpose of analysing the effects solely of Royalactin on the growth of insects.

Due to time constrains, I had to desist trying to purify Royalactin. Nevertheless, the work carried out in this chapter shows that the initial protocol obtained from Kamakura (2011) is not suitable for the expression of Royalactin. Once a suitable *E. coli* strain was selected, expression was fast and easily scalable.

Future work will have to include more troubleshooting of the purification efforts, my work did not contain a strong process evaluating the reason why the protein disappeared, which in turn led to abandoning this line of work due to the seeming impossibility of preparing pure Royalactin.

# *Chapter 4* Effects of 5-azacytidine and RG108, two DNA methyltransferase inhibitors on the parasitoid wasp *Goniozus legneri* and the cricket *Gryllus bimaculatus.*

## 4.1 Introduction

DNA methylation is the process in which a methyl group is added to a base in a DNA molecule. The predominant base found to be modified in higher eukaryotes is cytidine. This process is performed by a set of enzymes called DNA methyltransferases (DNMTs). The process is part of the epigenetic modification of the genome, that leads to mitotically stable changes of the expression of genes without changing the DNA sequence.

DNA methylation activity has been linked to many potential effects in insects: caste control (Kucharski et al., 2008, Amarasinghe et al., 2014, Wojciechowski et al., 2018), task division (Araujo & Arias, 2021), behaviour and longevity (Yan et al., 2015, Cardoso-Junior et al., 2018), sex allocation (Cook et al., 2015), limb regeneration (Hamada et al., 2015) and memory and learning processes (Biergans et al., 2015, Biergans et al., 2016, Li et al., 2017). Manipulating the level of overall methylation of one specific organism can give the means to controlling the formation of these phenotypes. In this chapter, I report on inhibiting general DNA methylation using two DNA methyltransferase inhibitors: 5-azacytidine and RG108.

Compounds of the family of the azacytidines were first developed as cytostatic agents and then characterised as DNA methyltransferase inhibitors in 1980 (Schapira and Arrowsmith, 2016). 5-azacytidine is mostly used as a cancer chemotherapeutic agent,

due to its capacity to modulate some of the tumour's phenotypes (Jones, 1985). 5-azacytidine is a nucleoside analogue with DNA methyltransferase inhibitor properties, as it acts as a cytosine substitute during DNA duplication, blocking the release of the enzyme. All cytosine methyltransferases, including DNMT3 normally use the methyl group from the S-Adenosyl methionine (SAM) donor and covalently attach it to the C-5 position of cytosines. The DNMT3 enzyme then releases the substrate and is ready for a new reaction. 5-azacytidine inhibits this enzymatic process: the nucleoside analogue can accept the methyl group but prevents the DNMTs to detach from the substrate; the DNMTs enzymes are trapped and then degraded. The degradation causes the depletion of DNMTs in the cell which in turn leads to a lower degree of methylated DNA and a higher expression of genes that have hypomethylated regulatory sequences, such as promoters and enhancers (Stresemann et al., 2006, Stresemann and Lyko, 2008). 5-azacytidine incorporation into the DNA is random and can affect different locations of the genome (Yang et al., 2010).

RG108 is a novel synthetic human DNMT1 inhibitor. It was designed to interact with the human enzyme's active site, but due to the conservation of *DNMT* genes in animals (Jurkowski and Jeltsch, 2011), it was shown to be effective also on other species (Sun et al., 2016, Cardoso-Junior et al., 2018). RG108 has no reported toxicity, contrary to 5-azacytidine, and its half-life is also longer (Brueckner et al., 2005).

In the study by Kucharski at al. (2008), the authors obtained queens with fully developed ovaries by silencing the expression of the DNA methyltransferase 3 (DNMT3) in newly hatched honey bee larvae. This approach works by decreasing the ability of the

organism to *de novo* methylate DNA, and therefore modifying the normal DNA methylation patterns that are formed during development. Methylation takes place on the cytosines by the enzymes DNMT, with DNMT1 being a maintenance DNMT, DNMT2 having a very low DNA cysteine methylating activity, and DNMT3 being a *de novo* DNMT (Lyko, 2018, Edwards et al., 2017).

As with the approach of feeding insects on royal jelly, silencing of DNA methyltransferases can be applied to a variety of insect species, even though not all insects express the same set of DNMT methyltransferases. Mammals express three isoforms, DNMT1, 2 and 3, as do honey bees (Wang et al., 2006) but other insects, such as *Drosophila*, only have a DNMT2 ortholog (Vieira et al., 2018).

In Kucharski et al.'s (2008) study, DNMT3 function in honey bees was decreased by using RNA interference (RNAi). In the initial experiments described in this chapter, 5-azacytidine was used as an alternative approach to RNAi. The hypothesis is that it should be possible to induce a reduction in cytosine methylation in insects by using a DNMT inhibitor such as 5-azacytidine or RG108. This would lead to a reduction in methylation, and possibly to a higher expression of genes expressing metabolic enzymes and the general growth of the organism.

This chapter's hypothesis was that by using DNA methyltransferase inhibitors I could achieve effects comparable to the ones that Kamakura (2011) obtained feeding Royalactin to insects. The aim was to feed or inject chemicals that inhibit the insects' DNA methyltransferase and record the changes in the insects' phenotype focusing specifically on their weight change.

This chapter investigates the effects of wide-range methylation inhibition on the genome of the parasitoid wasp *Goniozus legneri* Gordh (Hymenoptera: Bethylidae) and of the cricket *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae), particularly in the context of the possible generation of "queen like" effects, as obtained by Kucharski et al. (Kucharski et al., 2008).

## 4.2 Methods

### 4.2.1 5-azacytidine

5-azacytidine was injected (section 2.2.36) into the host of *G. legneri* (the rice moth, *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae) (section 2.2.35)) after it was paralysed by the wasp (via an injection of venom), and eggs had been laid upon it. I evaluated the weight change in the new generation of *G. legneri* wasps that developed by feeding on such hosts. All adult female offspring were weighed once they had died of old age, using an electronic balance (Precisa, model 2625MA, accuracy: 0.01 mg).

#### 4.2.1.1 Injections in *Goniozus legneri*

The wasp *Goniozus legneri* is a parasitoid that reproduces by laying its eggs on the paralysed bodies of caterpillars. At 27°C the eggs take one day to hatch and then the newly formed larvae bite into and anchor onto the host's integument to feed from it. Feeding lasts around three days then the larvae move away from the remains of the host, spin a cocoon and pupate. Pupae take another seven days to develop to adulthood(Figure 4 1).

Typically, only one or a few eggs in each clutch is male, with clutches ranging from 4-18 eggs (Khidr et al., 2013, Du et al., 2021). Males are the first to hatch (protandry) (Hardy et al., 2000) and are smaller than females. Males mate with the females within the brood and usually die three to four days after hatching. The experiments using *G. legneri* focused only on effects on female wasps due to their larger size and greater numbers; males were therefore not included in the data analysis.

For the experiments, I took advantage of the fact that the larvae feeding on the caterpillar for around three days. Therefore, the solution containing 5-azacytidine was injected directly into the body of the caterpillar after it was paralysed by the wasp and before it had eggs laid on it. My expectations were that once injected, the solution would spread into the caterpillar's body through the haemolymph, as shown by Goubault and Hardy (2007) who injected deuterium into caterpillars and detected it in adult *G. legneri* wasps that had fed on these caterpillars. Suitable volumes for injections, avoiding overflow, were identified heuristically, the initial volume was 10 µL but, since some liquid would usually spill out from the caterpillar, the volume was decreased to 5 µL but in turn the concentration was increased to keep the final dose constant. Injections followed the protocol described in section 2.2.36.

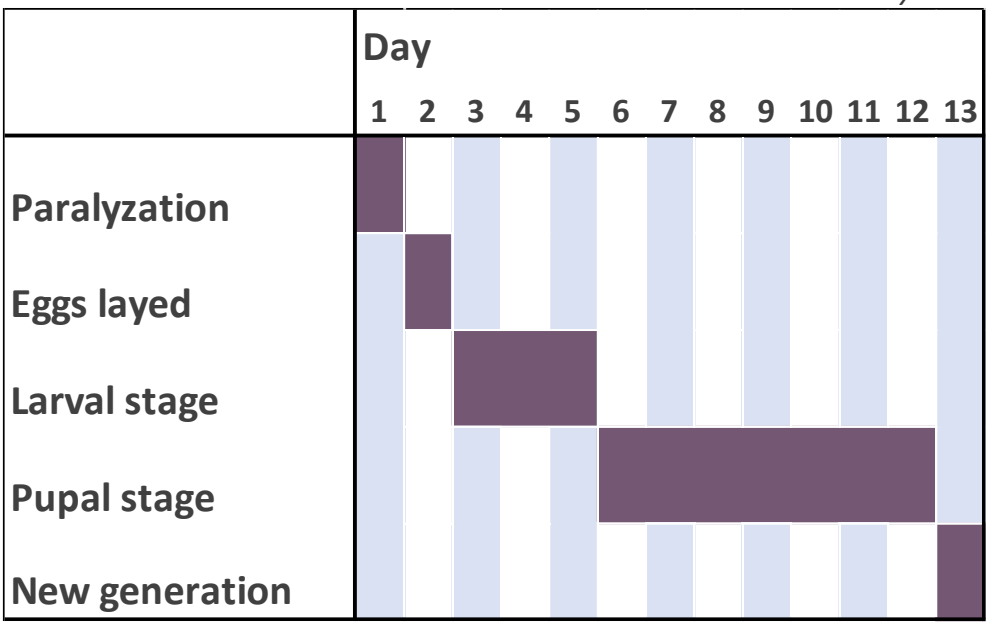



*Figure 4.1 Timing of offspring production by wasp G. legneri on the host C. cephalonica.*

#### 4.2.1.2 Solvents for 5-azacytidine solutions

5-azacytidine was received as a powder and was mixed with two different solvents: dimethyl sulfoxide (DMSO) in a pilot experiment, and phosphate buffered saline (PBS). DMSO was the initial choice as it is one of the manufacturer's suggested solvents, it is a cryoprotective agent and it is also a carrier of compounds into biological membranes (Sambrook and Russell, 2001, Egorin et al., 1998). However, all the pilot experiments that contained 5-azacytidine mixed with DMSO resulted in 100% mortality (Figure 4.2), with almost all G. legneri wasps dying during their larval stage, and only 6.2% able enter the pupa stage but without first producing cocoons. This led to a change of solvent to 0.1 M PBS (a saline solution), to mimic the pH and general body conditions of the caterpillar: mortality decreased sharply from 100% to 9% (Figure 4.2), which is within the range of laboratory estimates of the natural rate of egg-to-adult mortality, 7-12% (Khidr et al., 2013).

To calculate the treatments' final concentration, I rounded-up the volume of the hosts: the hosts' average weight was around 35 mg and set the volume at 35 mL. To measure *G. legneri*'s weight, wasps were left to die of old age and then weighed, this was due to the fact that female *G. legneri* wasps egg load varies during their lifetime, reaching a peak after around 9 days (Stokkebo and Hardy, 2000). Allowing them to die of old age reduces the egg load of adults (P. Pardo & I.C.W. Hardy pers. comm.) towards zero, thus reducing a source of weight variability among the adult *G. legneri* wasps.

### 4.2.2 RG108

RG108 was suspended in 100% ethanol to obtain a 10mM stock solution, as per manufacturer's recommendation. Working solution was prepared on the same day of the experiment by obtaining a 10µM solution using PBS. RG108 was tested on *Goniozus legneri* by injecting it into its host *Corcyra cephalonica*, as described in the previous paragraphs (section 4.2.1.1), and by direct contact of larvae with the solution. I evaluated the weight change in the new generation of *G. legneri* wasps feeding on it. It was tested on *Gryllus bimaculatus* using direct injections on fifth instar insects and the adults' weight was analysed.

#### *4.2.2.1* Treatment of *G. legneri*

I injected the solution containing RG108 directly into the body of *G. legneri*'s host *Corcyra cephalonica*. As with the previous experiments with 5-azacytidine, I used 5 µL of 10µM solution.

I also decided to trial a topical application, as it is a viable treatment on insects with a larval stage (Goewie and Beetsma, 1976, Leonardi et al., 1996, Pasquier and Charmillot,

2004, Pettis et al., 2004). The application was either performed by dropping 10µL of the solution on the larvae that were feeding on *C. cephalonica* or by immerging the host and the larvae in a tube containing the solution. After either treatment, the host and the larvae were put back into the culture room.

#### *4.2.2.2* Treatment of *G. bimaculatus*

Fifth instar *G. bimaculatus* crickets were obtained from Monkfield Nutrition Ltd. Injections into *G. bimaculatus* crickets were made using: a Hamilton 10 µL syringe, model 1701 SN SYR, cemented NDL, custom gauge (33), custom point style (4), angle (45), custom needle length (30mm). *G. bimaculatus* crickets were cooled in a freezer for 10 minutes and then put onto ice. The solution was injected in their abdominal body cavity between the third and the fourth abdominal segment. The injection technique is described in 2.2.38 and the location of the injection site was obtained from previous studies (Nakamura et al., 2007, Nakamura et al., 2008b, Dabour et al., 2011, Hamada et al., 2015).

## 4.3 Data analysis

All data obtained in the 5-azacytidine experiments were analysed using a general linear mixed modelling approach (Bolker et al., 2009): data on the weight of each female within a brood was analysed as the response variable and the identity of the brood was fitted as a random effect. The dose of 5-azacytidine was fitted as a fixed effect. As any relationship between dose and female weight may not be a straight line, a quadratic term (dose squared) was included in the model to assess curvilinearity.

Data obtained from the RG108 were analysed depending on the species. *Goniozus legneri* data were analysed using an ANOVA approach: female *G. legneri* wasps' weight was analysed as the response variable and treatment was used as fixed effect. *Gryllus bimaculatus* data was analysed using a two-way ANOVA to investigate the effects of sex and treatment. The criterion for significance in all analyses was $p<0.05$.

## 4.4 Results

### 4.4.1 Effects on *Goniozus legneri* wasp mortality of caterpillar manipulation and of 5-azacytidine injections

*Three injection treatments were used to evaluate the consequences of manipulating larvae-bearing-caterpillars on G. legneri wasp mortality rate (the percentage of eggs surviving to adulthood): a 4.4% w/v solution of DMSO dissolved in water, a 0.1 M solution of PBS and 5 different concentrations of 5-azacytidine dissolved in PBS: 50, 100, 150, 200 and 286 mM. The effect of these treatments on parasitoid developmental mortality were quantified by comparison to mortality in untreated G. legneri wasps (Figure 4.2). Mortality between treatments was analysed by logistic ANOVA and resulted in significant difference between treatments ($F_{7,72}$ = 55.47, p<.001). I performed a progressive aggregation of factors to reach the minimal adequate model (Crawley, 1993): all factors except DMSO can be grouped ($F_{1,78}$ = 375.06, p<0.001), with not significant reduction in the explanatory power of the model. This means that there is no difference of mortality between untreated G. legneri wasps and the use of PBS as a solvent for 5-azacytidine, or any concentration of the treatment. Effects of PBS injections are non-significant when compared to untreated G. legneri wasps ($F_{1,121}$ = 0.68, p=0.412).*

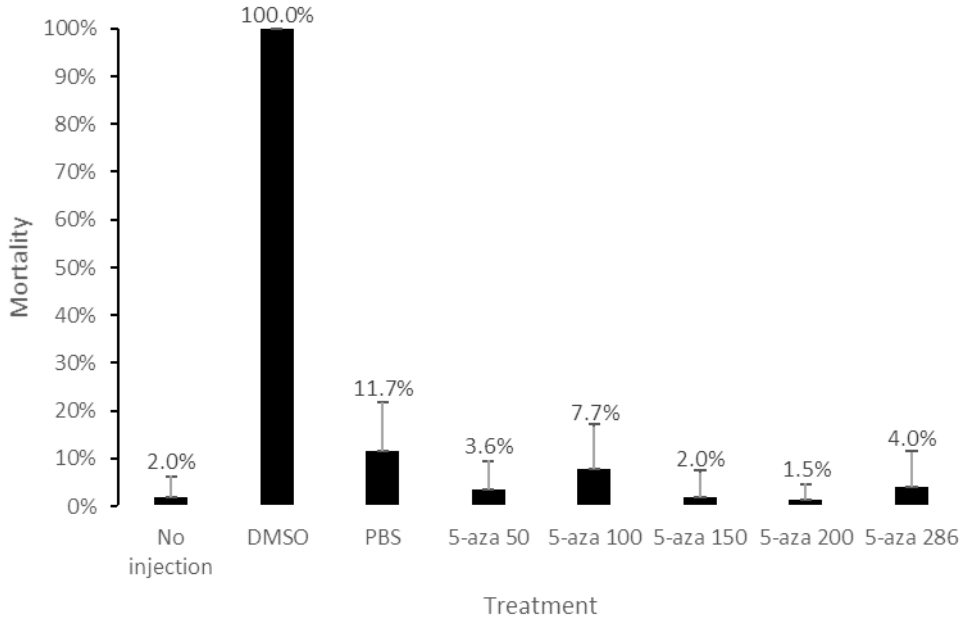



*Figure 4.2 Mortality of G. legneri wasp offspring after injecting different solutions into the host compared to no injection (control). DMSO concentration was 4.4% w/v, PBS was 0.1 M and 5-azacytidine was 50, 100, 150 200 and 286 mM. For each treatment n was no injection=10, DMSO=17, PPBS=3, 5-azacytidine-50=6, 5-azacytidine-100=17, 5-azacytidine-150=7, 5-azacytidine-200=11, 5-azacytidine-286=10. The experiment ran for 8 days, until adults emerged from the cocoons. Aggregation of factors show showed that there were no differences in mortality between treatments, except for DMSO for which mortality was significantly higher than in other treatments ($F_{1,78}$ = 375.06, p<0.001).*

The same experiment showed that adult *G. legneri* females varied in weight according to azacytidine concentration, with a peak between 5-azacytidine concentrations of 150 and 200 mM. Treatment had a significant effect on *G. legneri* wasp weight ($F_{5,421}$ = 18.17, *p*<.001) in a curvilinear relationship (quadratic term: $F_{1,25.3}$ = 7.73, *p*=0.010) (Figure 4.3). Weight increased positively with the increase of concentration reaching its maximum

around 175 mM 5-azacytidine and then decreases with higher concentrations. The average weight of non-treated *G. legneri* wasps was 0.20 mg, while the average weight for 150 mM and 200 mM concentrations was 0.28mg and 0.35mg respectively, which

correspond to between 140% and a 175% increase of weight. 530 *G. legneri* wasps from 63 caterpillars were used.

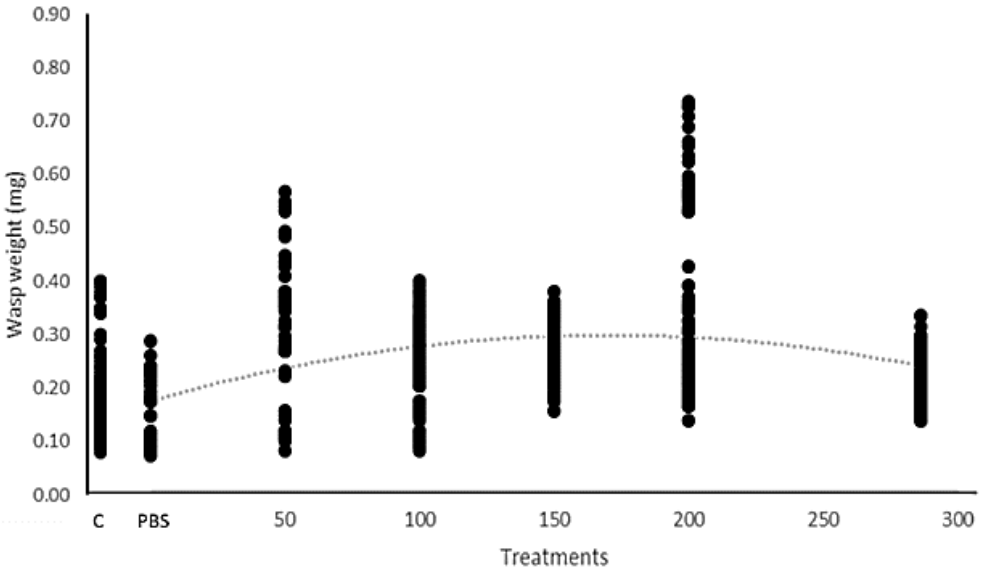



*Figure 4.3 Effects of injections of different concentrations of 5-azacytidine into C. cephalonica caterpillars on the weight of G. legneri adults emerging from those injected caterpillars. The experiment ran for eight days, until adults emerged from the cocoons. Individual data points represent the treated wasps' weight. C and PBS are two control treatments. C indicates that hosts were untreated (uninjected); PBS indicates that hosts were injected with PBS solution only. The 5-azacytidine solutions were dissolved in PBS, the concentrations were 50, 100, 150, 200 and 286 mM. The fitted quadratic regression line shows the curvilinear relationship between weight and dose, excluding untreated replicates ($F_{5,421}$ = 18.17, p<0.001).*

### 4.4.2 Effects of RG108 on *G. legneri*

Three experiments were performed on *G. legneri* using RG108. *G. legneri* wasp's host was injected with the solution; *G. legneri* wasp larvae attached to the host were

immersed in the solution; and finally, the solution was dripped onto the larvae. 523 larvae from 61 hosts were used. The outcome of these treatments on *G. legneri* wasp weight were analysed with an ANOVA, showing a significant effect ($F_{3,215}$ = 12.73, $p$<0.001). I performed a progressive aggregation of factors to reach the minimal adequate model (Crawley, 1993): "immersed" and "injected" treatments can be grouped with "untreated" ($F_{1,217}$ = 38.23, $p$<0.001), with no reduction in the explanatory power of the model. The only RG108 treatment that increased weight in *G. legneri* is by dripping the solution on larvae.

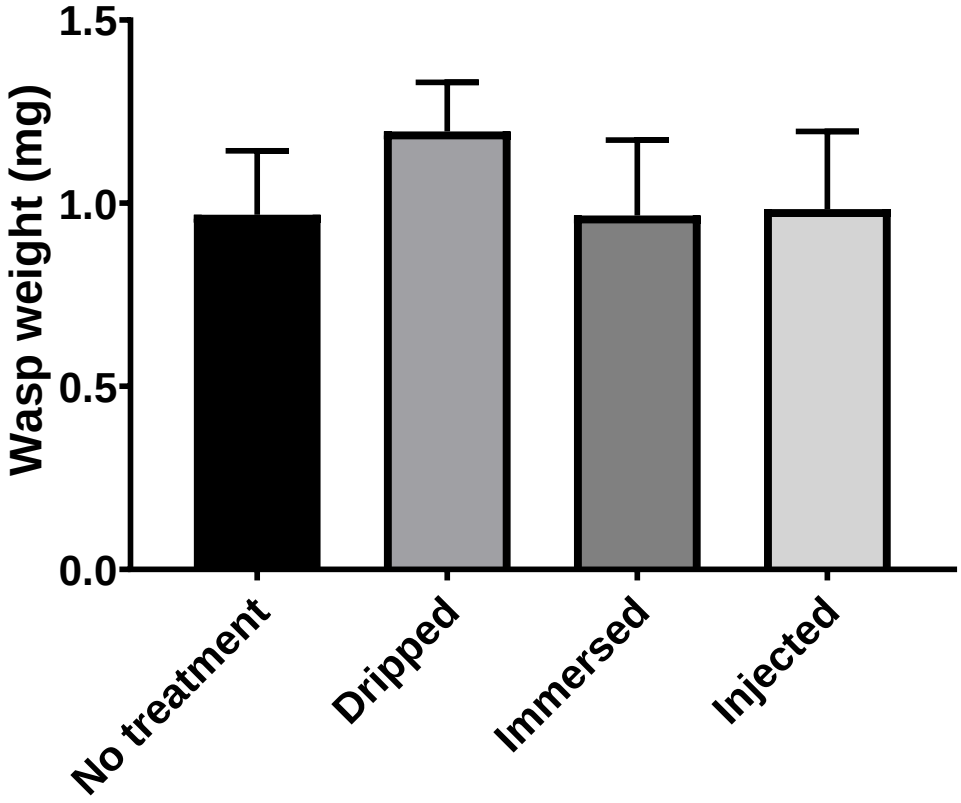



*Figure 4.4 Effects of topical application, immersion and injection into C. cephalonica caterpillars of RG108 on weight of G. legneri adults. All treatments used a solution of RG108 10µM. Untreated G. legneri wasps used as negative control. The experiment ran for eight days, until adults emerged from the cocoons. No treatment n=60 from 8 caterpillars, dripped n=32 from 8 caterpillars, immersed n=80 from 10 caterpillars, injected n=47 from 5 caterpillars.*

### 4.4.3 Effects of RG108 on *G. bimaculatus*

Three experiments were performed on *G. bimaculatus* using RG108. *G. bimaculatus* crickets were injected with two different volumes of a 10µM RG108 solution, 2 and 5µL. These *G. bimaculatus* crickets were compared to untreated *G. bimaculatus* crickets. In

the last experiment *G. bimaculatus* crickets were fed the solution ad libitum, with a PBS solution and untreated *G. bimaculatus* crickets as negative controls.

*G. bimaculatus* cricket nymphs do not exhibit sexual dimorphism, which only develop at the adult stage when females become bigger than males and the ovipositor develops. Injections were thus performed on unsexed nymphs and only once these had developed to adulthood was I able to form two groups to compare treatments taking insect sex into account. Consequently, there were not balanced numbers of males and females for every treatment type. A total of 62 crickets were analysed (females n=44, males n=18). Two-way ANOVA was used to calculate the effects of treatment and the influence on sex. Treatment was non-significant ($F_{4,52}$ = 0.76, $p$=0.554) as weight did not change significantly between treatments compared to untreated *G. bimaculatus* crickets. Sex effect on weight was significant ($F_{1,52}$ = 74.85, $p$<0.001) which is due to the *G. bimaculatus'* sexual body size dimorphism, with females having bigger bodies compared to males.

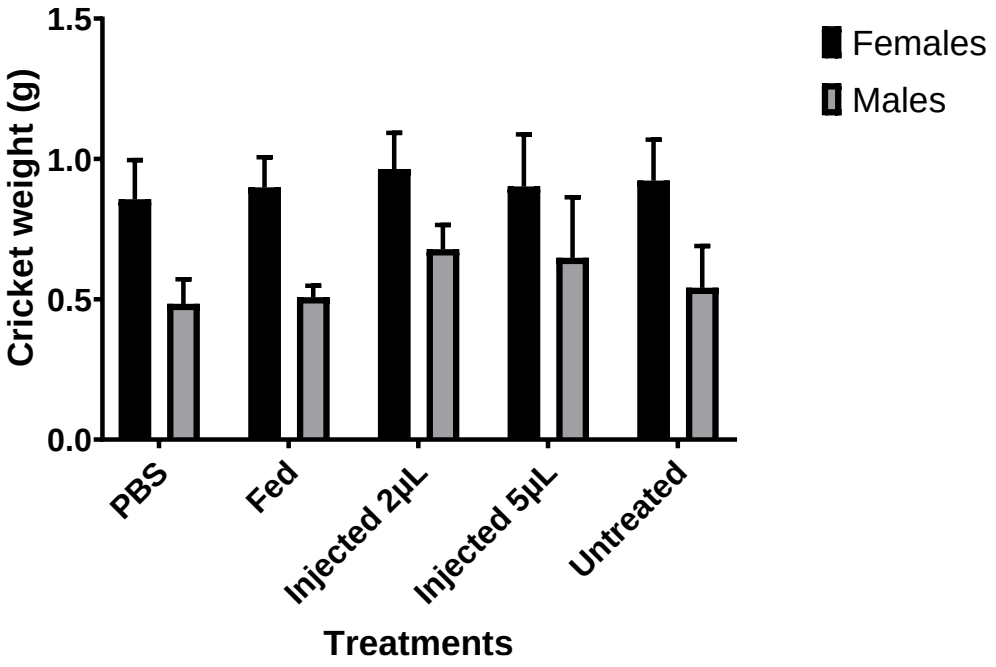



*Figure 4.5 Effects of feeding and injection of different volumes of RG108 on cricket weight. Untreated crickets used as negative control for injection experiment. All treatments used a solution of RG108 10µM. PBS, fed and untreated crickets used as negative controls. Black columns represent females, grey columns represent males. Treatment effects were non-significant ($F_{4,52}$ = 0.76, p=0.554), while sex effects on weight were significant ($F_{1,52}$ = 74.85, p<0.001). The experiment ran for 21 days, until nymphs moulted into adults. Untreated n=24 (16 Females, 8 Males), PBS n=11 (8F, 3M), fed n=10 (8F, 2M), injected 2µL n=7 (4F, 3M), injected 5µL n=10 (8F, 2M).*

## 4.5 Discussion

### 4.5.1 5-azacytidine experiments

The high mortality when the G. legneri wasps were fed 5-azacytidine diluted in DMSO or pure DMSO solution is most likely ascribed to the high DMSO toxicity: a study in honey bees showed how at a low intake concentration, queen fertility and egg hatching were not affected, but DMSO established an adverse effect on brood development (Milchreit et al., 2016). DMSO is also toxic to D. melanogaster (Cvetković et al., 2015). Both these prior studies used lower concentrations than what I used to prepare the original 5-azacytidine solution. Switching to PBS as the solvent for 5-azacytidine removed the toxicology problems of DMSO and allowed me to perform the experiments with a mortality 10 times lower (Figure

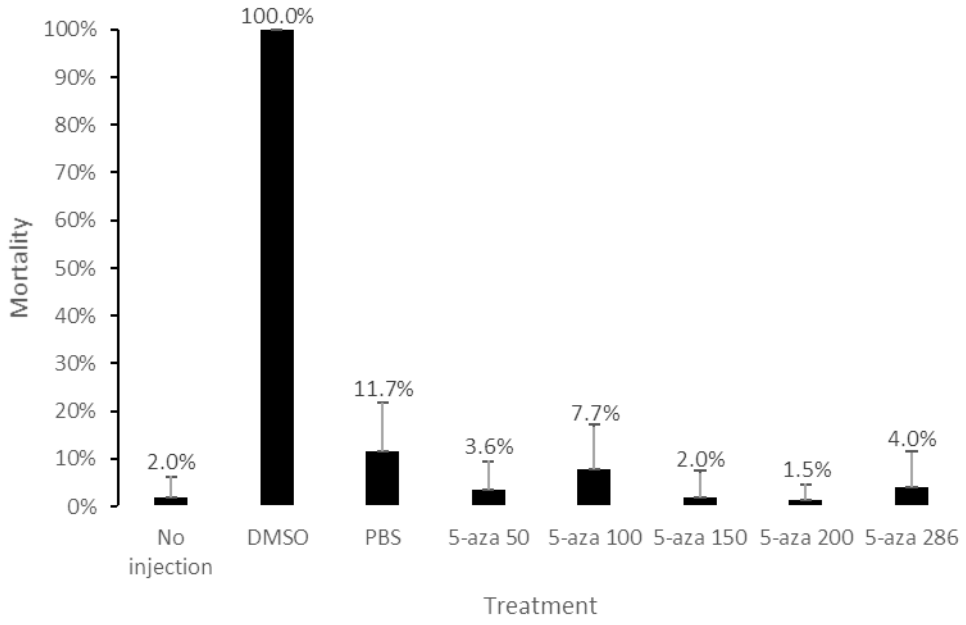



*Figure 4.2* 4.2).

One of the practical considerations surrounding the injection of hosts with 5-azacytidine solution is that its half-life is temperature dependent: 21 days at 4°C, 37 hours at 20°C and 7 hours at 37°C, but if the solution was injected at room temperature it still maintained its abilities to inhibit methylation (Stresemann and Lyko, 2008). This was

important because once the solution was injected into the caterpillar, the host was put in the culture room where the temperature was maintained constant at 27°C. The half-life of 5-azacytidine was shortened by the high culturing temperature but apparently was enough to induce a change in phenotype.

Another problem that I faced was the fact that I was introducing 5-azacytidine into *G. legneri* wasps via their diet, using their host as an intermediate step: there are no prior reports of the use of this method as the chemical is usually used in cell culture or in intravenous treatments. It is known that deuterium can be found in adult *G. legneri* wasps that as larvae have fed on caterpillars injected with them (Goubault and Hardy, 2007), but there are no previous data on 5-azacytidine. 5-azacytidine could be absorbed in the haemolymph and interact with the caterpillar before being ingested by *G. legneri* wasps. To be certain that the effects on *G. legneri* wasps is ascribed to 5-azacytidine more work needs to be done, specifically using global DNA methylation quantification, and comparing the treated *G. legneri* wasps to an untreated control.

The left side of the curve in Figure 4.1 could be ascribed to a small body-enlarging effect when the chemical's concentration is low. The decrease of weight on the right side could be attributed to the toxicity of 5-azacytidine when its concentration is too high, leading to a genome-wide deregulation of genes during development that could potentially be harmful. There are no studies about 5-azacytidine's specific toxicity on insects, but it is known to be toxic in high concentrations to mice and rats, and mammalian cells (Beisler, 1978, Holliday, 1987, Juttermann et al., 1994). The decrease of weight could also be related to a deleterious effect of overexpressing proteins (Moriya, 2015). Protein overexpression could cause an abnormal and toxic cellular environment, but this effect would not be reflected on mortality (Figure

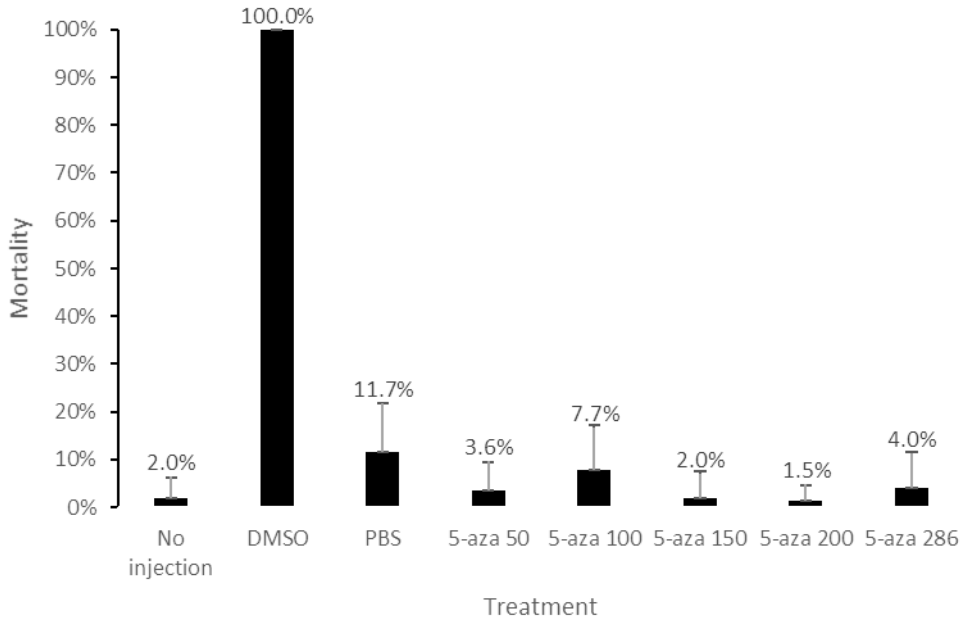



*Figure 4.2* 4.2).

From the results, I conclude that 5-azacytidine influences weight, but no analysis was performed on other parameters, and it is possible that other phenotypes were influenced. It is known that DNA demethylation increases longevity in eusocial insects

(Yan et al., 2015, Cardoso-Junior et al., 2018). It is also plausible that the over-expression of important genes, such as those involved in housekeeping or development, is controlled by feedback mechanisms such as ubiquitin-mediated proteolysis, to degrade the excess of proteins (Lecker et al., 2006). Expression of weight-increase genes might not be controlled as such, as fat storage is a mechanism that is positively selected, which might be the reason why using a non-cytotoxic chemical could increase the weight gain. A possible parallelism with queen bee development is that 5-azacytidine could cause larvae to feed more. To investigate this possibility, I analysed the host-weight-to-wasp-number ratio to infer a growth trend for each treatment (Appendix A). In all treatments except PBS, the number of *G. legneri* wasps increases with the increase in host weight (shown with a linear trendline). PBS negative trendline might be due to the low sample number. Conversely, *G. legneri* wasp weight does not show a correlation to host weight, as can be seen by the increasing and decreasing trendlines in Appendix B.

### 4.5.2 RG108 experiments

The expected results of using the methyltransferase inhibitor RG108 were to be comparable to what was obtained using 5-azacytidine, as the two chemicals follow different processes but have similar effects on the overall DNA methylation. Experiments with RG108 instead showed only one significant result on *G. legneri* wasps and no significant effects on *G. bimaculatus* crickets. The injection techniques utilised in this chapter have not previously been tried on insects. RG108 has only been used topically (Biergans et al., 2015, Cardoso-Junior et al., 2018) and found to negatively affect long-term memory and positively affect longevity. These studies confirmed that RG108 influences insects even though it is a synthetic human DNMT1 inhibitor.

Incidentally, the only positive result with RG108 was the drops treatment, but not the immersed treatment, which could also be regarded as a topical application.

My choice of dissolving RG108 in ethanol as a stock solution and then in PBS for the injections was based, respectively, on the manufacturer's instructions and the work of Brueckner et al. (Brueckner et al., 2005). Ethanol was deemed a safer solvent than DMSO due to previous results (Figure 4.2), although other studies (Biergans et al., 2015, Cardoso-Junior et al., 2018) used DMSO and dimethylformamide (DMF) as solvents with no apparent negative effects.

The two prior studies also used different concentrations of RG108 compared to the ones used in this chapter. Cardoso-Junior et al. (2018) used a 0.2mM solution, while Biergans et al. (2015) used a 2mM solution and both applied the solution topically to the thorax. The choice to use a 10µM solution followed the studies of Brueckner et al. (Brueckner et al., 2005) and Savickiene et al. (Savickiene et al., 2012)which showed, especially in the latter case, that concentration influences the speed of the methyltransferase inhibition rather than its effect.

## 4.6 Conclusions

The use of DNMT inhibitors, although it produced positive results, still needs to be further tested and, following this, a straightforward application technique developed that could be used in a commercial environment. Studies need to be carried out to find the best carrier in terms of avoidance of solvent poisoning in the experimental organism.

It must also be kept in mind that the effect of these compounds on the reduction of cytosine methylation is random (Brueckner et al., 2005, Stresemann and Lyko, 2008) and it could be affecting genes that were not analysed in these studies.

A more specific approach, such as the one described in the next chapter, can help to reduce the phenotypic uncertainties generated by general reduction of DNA methylation.

# Chapter 5 The effects of RNA interference on *Gryllus bimaculatus* and *Goniozus legneri*

## 5.1 Introduction

RNA interference (RNAi) acts by silencing the expression of targeted genes through the action of single or double stranded RNA (dsRNA) probes (Burand and Hunter, 2013). The probe enters the cells of the subject and becomes degraded by the ribonuclease enzyme Dicer into small RNA sequences of around 20 to 30 base pairs. These short interfering RNA (siRNA) sequences bind with Argonaute proteins (Hutvagner and Simard, 2008) native to the cell to form the RNA-induced silencing complex (RISC). The RISC complex exists naturally in all eukaryotic cells and its function is to perform RNA interference. Once the RISC complex is formed, it links to molecules of the cell's native messenger RNA (mRNA) that have the complementary nucleotide sequences of the siRNA. Once the RISC complex is linked to the mRNA, it cleaves the target RNA thanks to the protein Argonaute which is part of the RISC complex (Treiber et al., 2019).

In insects, RNAi is mainly being studied as a form of pest control (Huvenne and Smagghe, 2010, Kim et al., 2015, Abd El Halim et al., 2016, Laudani et al., 2017, Ghosh et al., 2017, Kolliopoulou et al., 2017, Luo et al., 2017,) but there are other applications of this technique. For instance, there have been studies on the effects of silencing certain genes on *G. bimaculatus* crickets' body size (Miyashita et al., 2016) or their ability to regenerate legs (Hamada et al., 2015, Nakamura et al., 2008a) and a number of physiological studies that include interference of the circadian rhythm (Moriyama et al., 2008) and interference on development-linked genes (Mito et al., 2007, Miyawaki et al., 2004).

The hypothesis addressed in this chapter is that knocking down the expression of genes involved in the development processes will reveal their function in increasing insect body weight. The aim is to explore effects of RNA interference in two different insect s species in different orders and with different life-histories, the parasitoid wasp *Goniozus legneri* and the cricket *Gryllus bimaculatus*, by targeting five genes involved in developmental processes to increase the adult insect body weight. This chapter describes the development of double stranded RNA probes for the five selected genes and reports on the attempts and observations to down-regulate their expression on the two insect species studied. The selected genes were: (1) *FoxO*, a gene that is known to enlarge body size in *G. bimaculatus* crickets (Dabour et al., 2011) but is also involved in longevity (Martins et al. 2016). Lowering or inhibiting the expression of *FoxO* aims to replicate the body-enlarging effects on crickets obtained by Dabour et al. (2011), and to establish whether these effects also manifest in wasps, given that increasing insect body size is one of the aims of the research presented in this thesis.

(2) *EGFR*, which has been tested on *G. bimaculatus* crickets and was shown to reduce body size (Dabour et al., 2011), but was not tested on parasitoid wasps. The study of the effects of *EGFR* knockdown links the experiments in this chapter with those presented Chapter 3, in which the effects of interfering with the EGFR signalling pathway was studied by feeding Royalactin to insects given that it is known that Royalactin acts on the EGF/EGFR pathway (Kamakura, 2011). Even though I was not able to feed the recombinant Royalactin that I produced to insects, if knocking down *EGFR* produces effects that are opposite to what Kamakura obtained by feeding Royalactin (i.e., increasing body size), then it would confirm that the EGFR pathway is involved in

controlling body size. This would also confirm that Royalactin has a specific role in inducing the queen bee phenotype and that the modifications are amenable to the bees' diet.

(3 & 4) *DNMT1* and *DNMT3*, genes involved in the methylation of DNA (Lyko, 2018), for which silencing has not previously been tested on insects. By knocking down these genes I aim to confirm the hypothesis explored in Chapter 4, in which I reduced DNA methylation by using inhibitors. Knocking down *DNMT1* and *DNMT3* links with experiments reported in Chapter 4 as the chemicals that were used in that chapter operate by inhibiting the effects of DNA methyltransferases. If the results obtained when injecting 5-azacytidine into *G. legneri* are due to the lower DNA methylation levels, then knocking out the genes that encode for the methylating enzymes should induce the same effects.

Finally, (5) *Myoglianin*, a gene involved in differentiation during embryogenesis and development of muscles, which RNA interference has only been tested before on *Drosophila* (Augustin et al., 2017). The hypothesis of RNAi-induced down-regulation of Myoglianin expression is that insects will produce more mass, as has been observed in *Drosophila* and in vertebrates with the homologue gene *Myostatin* (Lee and McPherron, 2001; Whittemore et al., 2003).

## 5.2 Methods

### 5.2.1 Extraction of total RNA

Frozen whole insects were suspended into RNA lysis buffer taken from Zymo ZR Tissue and Insect RNA MicroPrep kit, they were then processed in a homogeniser at highest

speed for around 20 seconds, until there were no visible pieces left (section 2.2.23). The homogenised samples were cleaned using phenol and chloroform (section 2.2.24) to remove proteins from the solution.

Total RNA was extracted from the organisms using the Zymo ZR Tissue and Insect RNA MicroPrep kit (section 2.2.25). The kit allows the extraction of RNA from a maximum of 10 milligrams of tissue: for *G. bimaculatus* crickets an entire 10-day old nymph was used, while for *G. legneri* wasps around 50 individuals were pooled for RNA extraction. The same technique was also performed at different *G. legneri* wasp developmental stages: larval, pupal, and adult. I found that the pupal stage is the only one in which *DNMT1* and *DNMT3* are expressed. I was unable to detect the expression of the genes of interest from samples deriving from larvae or adults: this might be because the genes are expressed at such low level that the PCR was unable to elongate any products.

### 5.2.2 Degenerated primer design

Neither the wasp *G. legneri* nor the cricket *G. bimaculatus* had had their whole genome sequenced at the time; for this reason, I did not have the sequence of most of the genes I wanted to study. *Goniozus legneri*'s genome was subsequently sequenced in 2018 (Kraaijeveld et al., 2018), while that of *G. bimaculatus*' has not yet been entirely sequenced. I obtained *G. bimaculatus* cricket's *FoxO* and *EGFR* primers sequences from Dabour et al. (Dabour et al., 2011), *DNMT1* was designed by Dr Reinhard Stöger, while all the other primers were produced by creating degenerated primers by aligning multiple complementary DNA (cDNA) sequences from different insect species to find conserved sequences in the genes (Appendix C). I found homologous cDNA sequences

of the gene I was interested in (Table 5.1 and Table 5.2) on the NCBI website (https://www.ncbi.nlm.nih.gov/) and I used Clustal Omega to align them (https://www.ebi.ac.uk/Tools/msa/clustalo/). Aligning and comparing the homologous sequences allowed me to highlight the conserved regions (Appendix 3). I then created primers from the conserved regions with the least nucleotide variations to obtain a low degeneracy for the primers. The degeneracy of a primer is the number of possible combinations that the specific sequence contains.

To help to visualise the degeneracy, I used the web-based application WebLogo (Crooks et al., 2004) and I obtained a logo for each primer (Figure 5.1). The logo is formed of letters that represent each base of the primer; every single primer position can have from zero to four stacked letters that represent how degenerated that position is. A blank space indicates that the program could not detect any conserved base in all the aligned sequences, which corresponds to the highest degeneracy. When multiple letters are stacked, the size of the letters reflects the frequency of those bases in the aligned sequences.

*Table 5.1 Multiple sequence alignments for FoxO and EGFR.*

| Gene | Species used |
|---|---|
| *FoxO* | XM_016913181.1 -> *Apis mellifera* |
| | NM_001316497.1 -> *Drosophila melanogaster* |
| | AB557980.2 GB -> *Gryllus bimaculatus* |
| | XM_008204819.2 -> *Nasonia vitripennis* |
| *EGFR* | XM_006560026.2 -> *Apis mellifera* |
| | NM_057410.4 -> *Drosophila melanogaster* |
| | AB300616.1 -> *Gryllus bimaculatus* |
| | XM_008215821.2 -> *Nasonia vitripennis* |

*Table 5.2 Multiple sequence alignments for Myoglianin and DNMT3.*

| | | |
|---|---|---|
| *Myoglianin* | LC128665.1 | -> *Gryllus bimaculatus* |
| | XM_016916400.1 | -> *Apis mellifera* |
| | XM_006621203.1 | -> *Apis dorsata* |
| | XM_016982834.1 | -> *Nasonia vitripennis* |
| | XM_015737513.1 | -> *Cephus cinctus* |
| | XM_015578631.1 | -> *Dufourea novaeangliae* |
| | XM_003402344.3 | -> *Bombus terrestris* |
| | XM_012390380.1 | -> *Bombus impatiens* |
| | XM_012284026.1 | -> *Megachile rotundata* |
| | XM_011259657.2 | -> *Camponotus floridanus* |
| | XM_011063332.1 | -> *Acromyrmex echinatior* |
| | XM_012204970.1 | -> *Atta cephalotes* |
| | XM_011638852.1 | -> *Pogonomyrmex barbatus* |
| | XM_011174553.1 | -> *Solenopsis invicta* |
| *DNMT3* | XM_008206224.2 | -> *Nasonia vitripennis* |
| | XM_011174635.1 | -> *Solenopsis invicta* |
| | XM_011271310.2 | -> *Camponotus floridanus* |
| | XM_012199554.1 | -> *Atta cephalotes* |
| | XM_011069215.1 | -> *Acromyrmex echinator* |
| | XM_015738858.1 | -> *Cephus cinctus* |
| | XM_015579479.1 | -> *Dufourea novaeangliae* |
| | XM_012281029.1 | -> *Megachile rotundata* |
| | XM_012386439.1 | -> *Bombus impatiens* |
| | NM_001353392.1 | -> *Bombus terrestris* |
| | NM_001190421.1 | -> *Apis mellifera* |
| | XM_006618195.1 | -> *Apis dorsata* |

The primers were designed to amplify gene portions between 300 and 600 base pairs. Once two suitable conserved regions per gene were found in the aligned sequences, the degenerate primers were created using IUPAC codes to represent the undefined

bases(Table 5.3). The degeneracy value represents the number of possible combinations that the primers represent (Table 5.4 and Table 5.5). The degeneracy value was calculated by multiplying the value of the bases: A, C, T and G are worth 1; R, Y, S, W, K and M are worth 2; B, D, H and V are worth 3; and finally, N is worth 4. The value of each base is dependent on the number of bases the base replaces (Table 5.3).

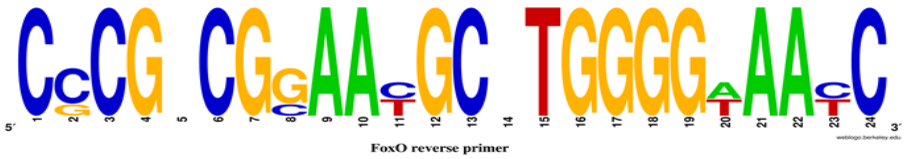
*Table 5.3 IUPAC names and values of the degeneracy code bases.*

| IUPAC nucleotide code | Base | Base value |
|---|---|---|
| A | Adenine | 1 |
| C | Cytosine | 1 |
| G | Guanine | 1 |
| T (or U) | Thymine (or Uracil) | 1 |
| R | A or G | 2 |
| Y | C or T | 2 |
| S | G or C | 2 |
| W | A or T | 2 |
| K | G or T | 2 |
| M | A or C | 2 |
| B | C or G or T | 3 |
| D | A or G or T | 3 |
| H | A or C or T | 3 |
| V | A or C or G | 3 |
| N | any base | 4 |

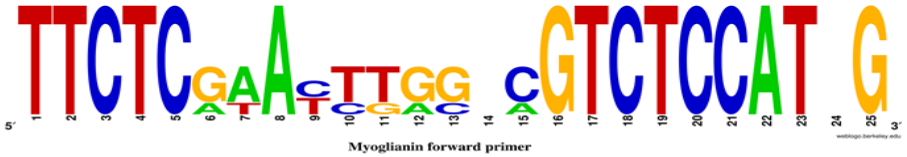
The lower the degeneracy the more effective the degenerate primer will be because it will contain a lower number of combinations representing unique sequences. If the degeneracy is high, the concentration of the correct combination could be so low that elongation could be impossible to achieve. This is the case for three of the degenerated primers I designed: *Myoglianin*, *DNMT1* and *DNMT3* did not produce any results in the PCR for either *G. bimaculatus* crickets or *G. legneri* wasps. I thus decided to reduce the degeneracy of *Myoglianin* and *DNMT3* by excluding from the uncertain positions those bases that had a very low frequency in the aligned sequences. I was able to reduce

*DNMT3*'s forward primer degeneracy from 768 to 8, *DNMT3*'s reverse primer degeneracy from 6144 to 32, *Myoglianin*'s forward primer degeneracy from 576 to 32 and finally, *Myoglianin*'s reverse primer degeneracy from 4096 to 8 (Table 5.7).

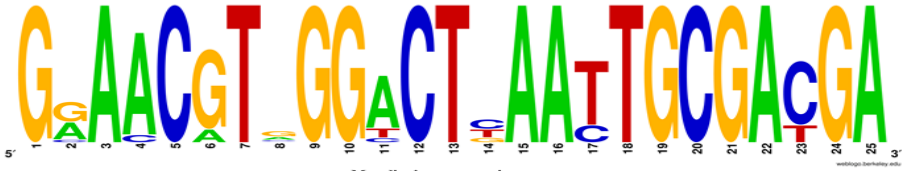
FoxO forward primer



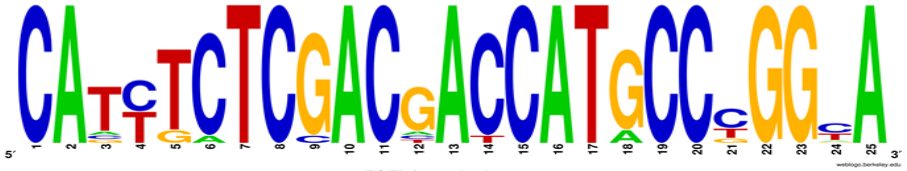
FoxO reverse primer



Myoglianin forward primer



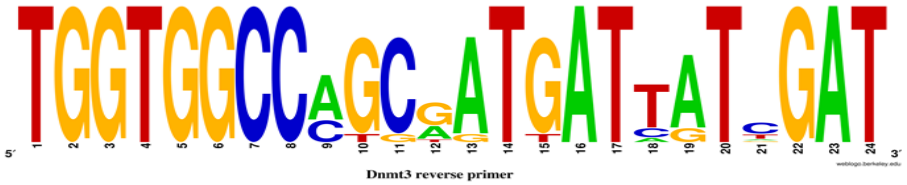
Myoglianin reverse primer



EGFR forward primer

EGFR reverse primer

Dnmt3 forward primer



Dnmt3 reverse primer

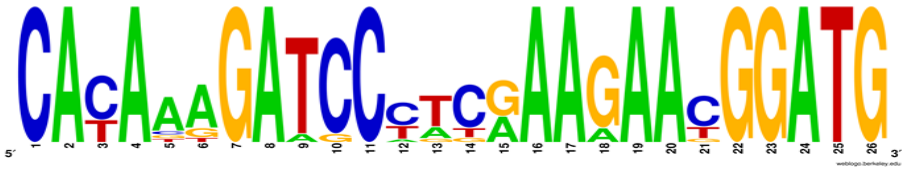


*Figure 5.1 Logos created with WebLogo for forward and reverse primers for FoxO, EGFR, Myoglianin and DNMT3 created with multiple aligned sequences. The letters represent each base of the primer and their size shows how degenerated that position is. A blank space indicates that the program could not detect any conserved base in all the aligned sequences. When multiple letters are stacked, the size of the letters reflects the frequency of those bases in the aligned sequences.*

*Table 5.4 Degenerate primers sequences and degeneracy values for FoxO, EGFR, DNMT1, DNMT3 and Myoglianin.*

| **Oligonucleotides** | **Sequence (5'->3')** | **Degeneracy** |
|---|---|---|
| *FoxO* F | CSCGNCGSAAYGCNTGGGGWAAYC | 512 |
| *FoxO* R | TTCTCRWAYYKRSDMGTCTCCATBG | 2304 |
| *EGFR* F | TACACSAACTGYACBTAYGTBGA | 72 |
| *EGFR* R | GCGCARAAVAGRTGRCARCAYTC | 96 |
| *DNMT1* F | YGGWGGDHVBGGWVDWMRHAAR | 279936 |
| *DNMT1* R | HHDNGRRTYHGMNGTYTCDCCNARHAYDG | 746496 |
| *DNMT3* F | TGGTGGCCMKSRRTKATHRTYGAT | 768 |
| *DNMT3* R | CAYANDGAWSCYWYRAARAAYGGATG | 6144 |
| *Myoglianin* F | GRAMCRTRGGHCTBAAYTGCGAYGA | 576 |
| *Myoglianin* R | CANYKMTCSACNAYCATRCCYGGYA | 4096 |

*Table 5.5 Modified degenerate primers sequences and degeneracy values for DNMT3 and Myoglianin.*

| **Oligonucleotides** | **Sequence (5'->3')** | **Degeneracy** |
|---|---|---|
| *DNMT3* F | TGGTGGCCMGCRATGATTATYGAT | 8 |
| *DNMT3* R | CAYAAAGATCCYWCRAAGAAYGGATG | 32 |
| *Myoglianin* F | GRAACGTRGGACTYAAYTGCGAYGA | 32 |
| *Myoglianin* R | CATYTCTCGACGACCATGCCYGGYA | 8 |

*Table 5.6 Length of degenerate primer and their amplicon.*

| Degenerate primer | Length (in base pairs) | Amplicon length |
|---|---|---|
| *FoxO* F | 24 bp | 325 bp |
| *FoxO* R | 25 bp | 325 bp |
| *EGFR* F | 23 bp | 551 bp |
| *EGFR* R | 23 bp | 551 bp |
| *DNMT1* F | 22 bp | 615 bp |
| *DNMT1* R | 21 bp | 615 bp |
| *DNMT3* F | 24 bp | 578 bp |
| *DNMT3* R | 26 bp | 578 bp |
| *Myoglianin* F | 25 bp | 319 bp |
| *Myoglianin* R | 25 bp | 319 bp |

### 5.2.3 Creation of complementary DNA from the total RNA

Total RNA was extracted from whole insects: single 10-day-old *G. bimaculatus* crickets were homogenised, while around 50 adult *G. legneri* wasps were pooled and then homogenised (section 2.2.23). The homogenised samples were centrifuged to remove the pellet. The total RNA was extracted using phenol/chloroform, following the protocol in section 2.2.24. The cDNA was produced (section 2.2.25) starting from the extracted total RNA using two different reverse transcriptases: Transcriptor reverse transcriptase (Sigma-Aldrich), and SuperScript III reverse transcriptase (Thermo Fisher Scientific). These reverse transcriptases have a very similar protocol (section 2.2.26), involving a reverse transcriptase that links to one primer to elongate a portion of the RNA into DNA. All efforts to start the reverse transcription process using the degenerate primers were unsuccessful but I was able to produce cDNA by using random primers in place of the degenerate primers. Random primers are random hexa-nucleotides that, when used to produce cDNA, generate short DNA sequences scattered all over the total RNA. Having to use random primers added a step to the procedure but allowed us to create a "total cDNA pool" that could be used with any degenerate primer pair in the PCR reactions.

### 5.2.4 Elongation of the selected sequences using degenerate primers and cloning of the genes' sequences into pCR4-TOPO vector

Separate PCR reactions were set up to elongate each gene for both species. The general PCR conditions are given in Chapter 2 (section 2.2.27) but the annealing temperature was modified for each reaction depending on the primers sequence. The DNA products from the PCR reactions were run on 1.5% agarose gels with the 2-Log DNA Ladder (NEB) to compare the expected size of the fragments (section 2.2.16). The expected size of the fragments was obtained from the homologous genes' multiple alignment: I averaged the number of bases for each gene I compared to have a general idea of the size I was looking for. If the fragments were of the expected size, they were excided from the gel. The DNA was then extracted from the gel using the QIAEX II gel extraction kit (QIAGEN) (section 2.2.28) and it was then ligated into the pCR4-TOPO vector (Thermo Fisher Scientific) (section 2.2.29) and then transformed into Stable Competent *E. coli* (NEB) (section 2.2.4). The transformed cells were plated on Carbenicillin Petri dishes containing X-gal to highlight the cells that contained the vector. The successfully transformed cells are recognised using the blue and white screening. Blue colonies are the result of the metabolization of X-gal, which can only occur if the cell can produce the enzyme β-galactosidase. A property of this enzyme is that it can be functional even if two parts of its protein sequence are produced by two separate DNA units, together encoding full-length β-galactosidase: the bacterial genome encodes one part of the gene and the vector encodes the other. The portion of the enzyme in the vector contains a multiple cloning site inside it (Figure 5.2); when the insert is correctly cloned into the vector, the coding sequence of the enzyme is disrupted. This halts the

formation of the β-galactosidase's second portion, thus rendering the cell unable to create a functional enzyme able to process X-gal. This method allows to visual discrimination between colonies where the transformation was successful and those in which it was not.

The cells that showed white colouration were selected and used to inoculate liquid cultures which were then used to produce minipreps using the Zyppy plasmid miniprep kit (Zymo Research) (section 2.2.30). Minipreps are used to isolate the plasmid from the bacteria, providing a safe way to conserve the target sequence and to transform new bacteria to produce larger quantities of the vector itself. The pCR4-TOPO vector has two sequencing primers flanking the insert sequence called M13 (Figure 2.2), this allowed the PCR reaction to run using those primers and then the products to be run on agarose gel to check the presence and size of the insert to confirm the success of the cloning. It also allowed us to send the vector to have the insert sequenced using sequence-specific primers (section 5.2.5).

### 5.2.5 Sequencing of PCR products sequences

Five microliters of the mini-prep solution for each gene were sent to Source BioScience (Nottingham, UK) to be sequenced. The results were presented in the form of a series of peaks each representing a single base; the quality and clarity of the peaks is linked to the quality and concentration of the DNA. I compared the sequences I obtained from Source BioScience to the NCBI nucleotide database using the Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), this online tool produces a list of the similarity

between the sequence obtained and any gene in the database. If homologous genes have a high similarity, it indicates the primers are elongating the correct genes.

The degenerate primers were successful in elongating sequences for three *G. legneri* wasp genes (*FoxO*, *EGFR* and *Myoglianin*) and two *G. bimaculatus* cricket genes (*FoxO* and *EGFR*).

### 5.2.6 Creating primers from *G. legneri* wasp genome

In 2018, Kraaijeveld et al. (Kraaijeveld et al., 2018) sequenced the genome of *G. legneri* and published an online database (http://parasitoids.labs.vu.nl/parasitoids/). The database includes a blasting tool to compare sequences to the whole *G. legneri* genome or only to the transcripts, which I used to compare the DNA segments that I obtained using the degenerate primers to check if the sequences matched with any *G. legneri* wasp gene. For *FoxO*, *EGFR* and *Myoglianin* the match was perfect, and the obtained PCR product had the same sequence as the cDNA that I blasted against. The primers for *DNMT1* and *DNMT3* were instead redesigned using the sequence from the *G. legneri* wasp genome, since all our PCR attempts were unsuccessful to produce any elongation.

### 5.2.7 Species-specific primer design

The new species-specific primers, except for *G. legneri* wasp's *DNMT1* and *DNMT3*, were designed based on the DNA sequences obtained from Source BioScience, by modifying the degenerate bases of the degenerate primers to the sequenced ones. Through this process, I was able to design all five primers (forward and reverse) for *G. legneri* wasps (Table 5.7).

*FoxO* and *EGFR* primers for *G. bimaculatus* cricket were obtained from Dabour et al. (Dabour et al., 2011). I managed to amplify *G. bimaculatus* cricket *DNMT1* using *G. legneri* wasp species-specific primers (Table 5.8).

I hypothesised that amplification from the *DNMT1* primer may be due to the DNMT gene family being generally conserved in animals and the same primers can amplify orthologous genes in different species (Jurkowski and Jeltsch, 2011). In contrast with *DNMT1*'s results, using *G. legneri* wasp's *DNMT3* species-specific primer in a PCR with *G. bimaculatus* cricket's DNA did not produce any amplification.

*Table 5.7 G. legneri wasp species-specific primers.*

| **Oligonucleotides** | **Primer sequence (5'->3')** | **Bases** | **PCR product sequence length (bp)** |
|---|---|---|---|
| *FoxO* F | CGCGGCGGAATGCGTGG | 17 | 362 |
| *FoxO* R | TTCTCGAATCGGGGCGTCTC | 20 | 362 |
| *EGFR* F | TACACGAACTGTACCTACGTGG | 22 | 591 |
| *EGFR* R | GCGCAGAAGAGGTGGCAG | 18 | 591 |
| *DNMT1* F | CGAAGAAGATCCTTCTGCAGAGGG | 24 | 416 |
| *DNMT1* R | CTGTGAATCTAGACAACCCTTGTGG | 25 | 416 |
| *DNMT3* F | AGATATTATTCAAAGATCCGTGGG | 24 | 439 |
| *DNMT3* R | TAGCCAGACTTAAGTCATTACAGGG | 25 | 439 |
| *Myoglianin* F | GGAACGTGGGCCTTAACTGC | 20 | 359 |
| *Myoglianin* R | CATTTATCGACTATCATGCCTGG | 23 | 359 |

*Table 5.8 G. bimaculatus cricket species-specific primers.*

| **Oligonucleotides** | **Primer sequence (5'->3')** | **Bases** | **PCR product sequence length (bp)** |
|---|---|---|---|
| *FoxO* F | CGCGGCGGAACGCGT | 15 | 365 |
| *FoxO* R | TTCTCGTACCTGGGCGTCTCCATTG | 25 | 365 |
| *EGFR* F | TACACGAACTGTACGTATGTGGA | 23 | 591 |

| | | | |
|---|---|---|---|
| *EGFR* R | GCGCAGAACAGGTGGCAGCATTC | 23 | 591 |
| *DNMT1* F | CGAAGAAGATCCTTCTGCAGAGGG | 24 | 376 |
| *DNMT1* R | CTGTGAATCTAGACAACCCTTGTGG | 25 | 376 |

### 5.2.8 Creating double stranded RNA probes

I used the MEGAscript T7 kit (Thermo Fisher Scientific) (section 2.2.31) to transcribe DNA into RNA, but first I had to add the T7 promoter sequence to the primers: an extension of 20 bases that the T7 polymerase needs to attach to the DNA to start its transcription activity. With the new primers' pairs, I ran the previously produced PCR products in new PCR reactions (section 2.2.27.3) and added the T7 promoter to their sequence.

I simultaneously produced both sense and antisense RNA strands and obtained dsRNA by heating the solution and decreasing the temperature slowly, allowing the strands to anneal. DNA was removed using the kit's TURBO DNase (Thermo Fisher Scientific) and then the dsRNA was precipitated using salts and ethanol and reducing the temperature of the solution. The dsRNA was pelleted and then resuspended in the injection buffer (section 2.2.31). The purity and the concentration of the RNA was measured using a spectrophotometer (section 2.2.32) with two different readings: 260 nm for proteins and 280 nm for RNA. The ratio of the two readings indicates how pure the RNA is, with the lowest content of proteins being the purest.

Table 5.9 shows the molecular weight, concentration, and molarity of *FoxO*, *EGFR*, *Myoglianin*, *DNMT1* and *DNMT3* dsRNA for *G. legneri*. Table 5.10shows the second batch I produced for some of *G. legneri's* dsRNA to try to increase the final concentration to allow me to inject smaller volumes. To achieve this, I modified the

protocol for the MEGAscript T7 (Thermo Fisher Scientific) (section 2.2.31): I upscaled the reaction from 20 to 100 µL but only resuspended the final pellet in half the injection buffer (50 µL instead of 100 µL). Table 5.11 shows the molecular weight, concentration, and molarity of *FoxO*, *EGFR* and *DNMT1* dsRNA for *G. bimaculatus*.

*Table 5.9 Molecular weight, concentration and molarity of the first batch of FoxO, EGFR, Myoglianin, DNMT1 and DNMT3 dsRNA for G. legneri.*

| dsRNA | Molecular weight | Concentration | Molarity |
|---|---|---|---|
| *FoxO* | 210173.7 Da | 2.26 µg/µL | 10.75 µM |
| *EGFR* | 414276.4 Da | 3.05 µg/µL | 7.36 µM |
| *Myoglianin* | 206525.9 Da | 5.17 µg/µL | 25.03 µM |
| *DNMT1* | 244619.7 Da | 3.28 µg/µL | 13.41 µM |
| *DNMT3* | 324557.9 Da | 3.29 µg/µL | 10.14 µM |

*Table 5.10 Molecular weight, concentration and molarity of the second batch of FoxO, EGFR, DNMT1 and DNMT3 dsRNA for G. legneri.*

| dsRNA | Molecular weight | Concentration | Molarity |
|---|---|---|---|
| *FoxO* | 210173.7 Da | 11.3 µg/µL | 53.77 µM |
| *EGFR* | 414276.4 Da | 9.42 µg/µL | 22.74 µM |
| *DNMT1* | 244619.7 Da | 9.18 µg/µL | 37.53 µM |
| *DNMT3* | 324557.9 Da | 8.20 µg/µL | 25.27 µM |

*Table 5.11 Molecular weight, concentration and molarity of FoxO, EGFR and DNMT1 dsRNA for G. bimaculatus.*

| dsRNA | Molecular weight | Concentration | Molarity |
|---|---|---|---|
| *FoxO* | 211577.2 Da | 8.10 µg/µL | 38.28 µM |
| *EGFR* | 356675.9 Da | 7.78 µg/µL | 21.80 µM |
| *DNMT1* | 225396 Da | 4.72 µg/µL | 20.94 µM |

### 5.2.9 DsRed as a negative control

I used the gene for the protein DsRed as a negative control because it is a gene that is not expressed in insects. DsRed is also known as red fluorescent protein, the gene was originally isolated from *Discosoma*, a genus of cnidarians. When the dsRNA is injected into animals that do not possess the gene, there should be no effect.

I obtained the pDsRed-Express Vector (Clontech) containing the *DsRed* gene from Dr Simon Welham (School of Biosciences, University of Nottingham, UK). The sequence was already known, and I designed the primers containing the T7 sequence to be able to create the dsRNA. I chose two primers that amplify a 394 base pairs sequence. The dsRNA production process followed the same steps as for the other genes (section 2.2.31). *DsRed* dsRNA has 394 bases.

*Table 5.12 DsRed primers.*

| **Oligonucleotides** | **Primer sequence (5'->3')** | **Bases** | **PCR product sequence length (bp)** |
|---|---|---|---|
| *DsRed* F | CGACTACAAGAAGCTGTCCTTC | 22 | 394 |
| *DsRed* R | TTGGAGTCCACGTAGTAGTAGCC | 23 | 394 |

### 5.2.10 dsRNA degradation at room temperature

The dsRNA solutions were injected into the subjects following the same method used in Chapter 4 (sections 2.2.36 and 2.2.38). The injections were performed at room temperature, but the treated insects were stored in the culture rooms at 26°C. I analysed the stability of a dsRNA solution at room temperature for one week to estimate the rate of degradation of the sequences. This procedure was carried out to

rule out the possibility that any lack of effect might be due to the degradation of the RNA during long exposure to warm temperatures.

After 6 days at room temperature, the dsRNA band intensity was as strong as the freshly produced RNA. Thus, products are free from RNases and that room temperature does not promote degradation process.

Presence of RNases in the dsRNA solution is not the only potential cause of degradation: analysis of the output of RNA interference in 37 different species belonging to five insect orders found that different insects have different dsRNA degradation abilities and that the different ways that dsRNA is supplied to the insect influences its processing (Singh et al., 2017). Injecting dsRNA into members of the Orthoptera (the taxonomic order to which *G. bimaculatus* also belongs) yielded good results but these differed across species. Injecting dsRNA into lepidopterans (the insect order to which *C. cephalonica*, the host of *G. legneri*, also belongs) did not seem to be effective, due to both degradation by RNases and by inefficient processing of dsRNA to siRNA. Unfortunately, Singh et al. (Singh et al., 2017) did not study any hymenopterans but I assumed that RNA interference works in *G. legneri* due to other studies on parasitoid *G. legneri* wasps (Lynch and Desplan, 2006).

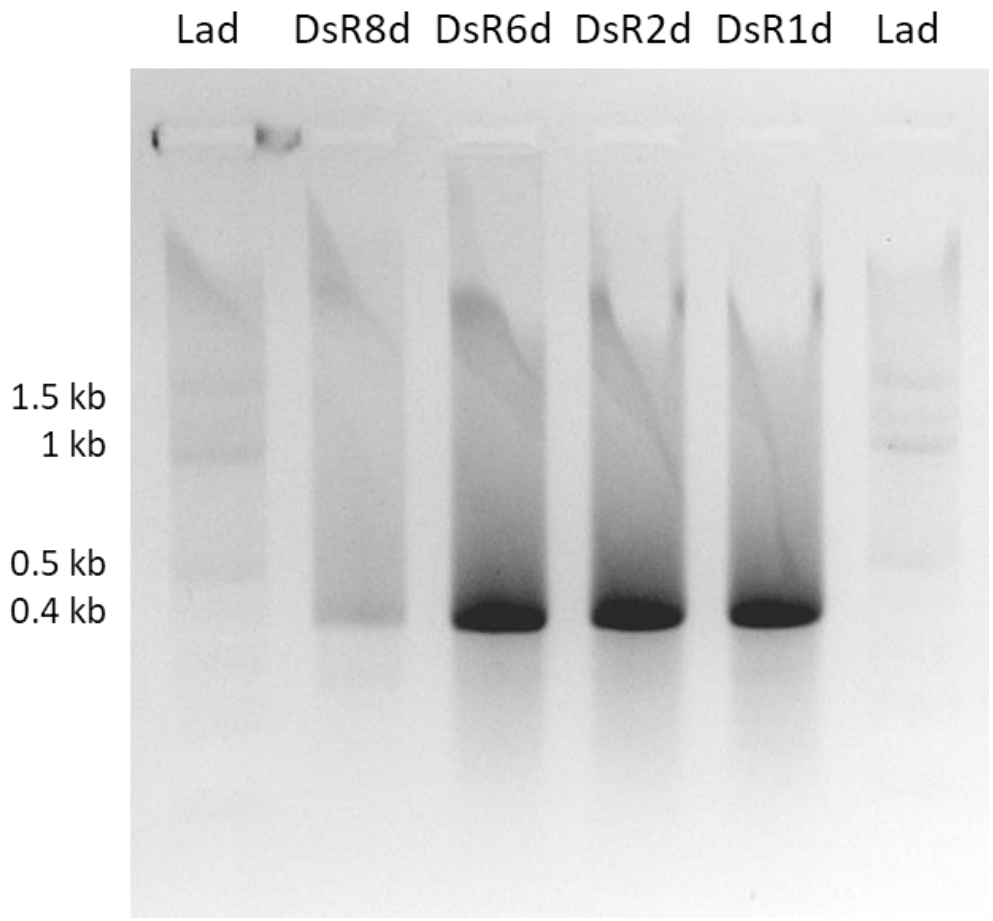



*Figure 5.2 dsRNA degradation test. DsRed dsRNA was sampled and kept at room temperature for 8, 6, 2 and 1 days to evaluate the rate of any degradation. Ladder is 2-log DNA ladder (NEB).*

### 5.2.11 Injections in *G. legneri*'s host, *Corcyra cephalonica*

Trials of injections into developing *G. legneri* wasps were made using a similar method to the one reported by Lynch and Desplan (2006), in which they injected the wasp *Nasonia vitripennis* using a self-made apparatus. PBS was injected using pulled glass needles, these needles were made using calibrated glass capillary tubes pulled over a flame. As RNAi sensitivity may be affected by developmental stage (Liu et al., 2010), I tried injecting into both pupae and larvae (section 2.2.34), but in most trials, they were

too thick to pierce the insect's body, with the pupa dying soon after injection has been achieved. Injecting into larvae was also problematic because, once pierced, the pupa's innards would spill out due to a positive pressure inside the body (Figure 5.3).

I therefore injected the paralysed host *C. cephalonica* as means of transferring dsRNA to subsequently feeding *G. legneri* wasp larvae (section 4.2.1.1, protocol in section 2.2.36). Expectations of injecting RNA into the hosts were that the probes would distribute in the host's haemolymph, being subsequently eaten by the *G. legneri* wasp larvae without being degraded in their digestive system and, finally, have an effect. This method relies on there being little interaction between the host and the dsRNA (inefficient processing of injected dsRNA is observed in other lepidopterans (Singh et al., 2017, Shukla et al., 2016)).

All female *G. legneri* wasps were put into the freezer at -20°C as soon as they emerged from the cocoon and then weighed using an electronic balance (Precisa, model number 2625MA, accuracy: 0.01 mg). Males were ignored, as explained in section 4.2.1.1.

### 5.2.12 Injections in *G. bimaculatus*

Fifth instar *G. bimaculatus* cricket nymphs were selected to be injected based on the work of Dabour et al. (Dabour et al., 2011), as it is a good compromise between a longer experiment when using the third instar, and a shorter exposure to the dsRNA when using the eighth instar. Injections were performed as explained before in Chapter 4, section 4.2.2.2.

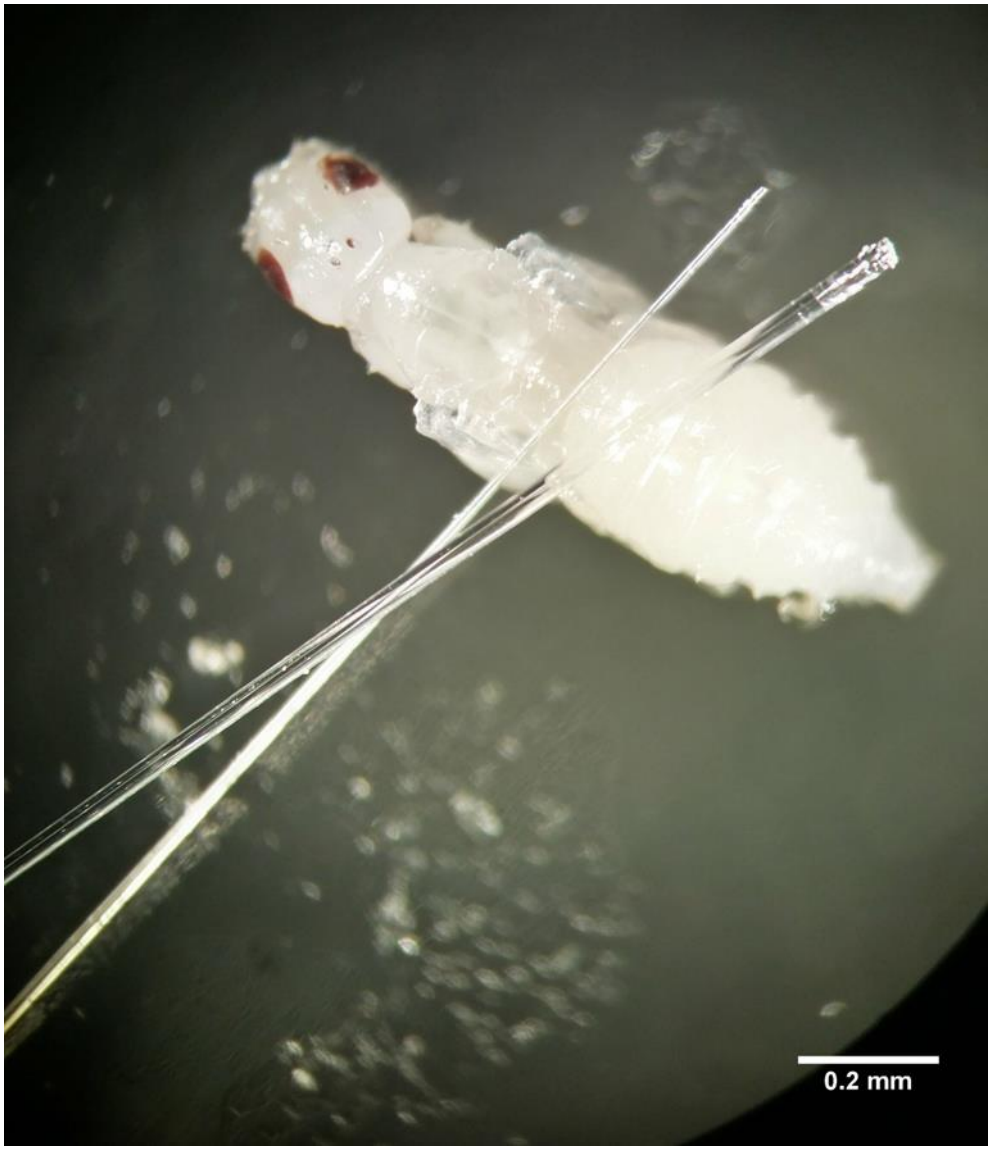



*Figure 5.3 G. legneri pupa removed from their cocoon, compared to the size of two pulled glass needles.*

All male and female *G. bimaculatus* crickets were put in the freezer as soon as they moulted into adults and then weighed using an electronic balance (Precisa, model number 2625MA, accuracy: 0.01 mg).

### 5.2.13 Data analysis

All data obtained from RNA interference experiments were analysed using a generalised linear mixed modelling approach (Bolker et al., 2009). Data on the weight of each female

*G. legneri* wasp within a brood was analysed as the response variable and the identity of the brood was fitted as a random effect. The dose of 5-azacytidine was fitted as a fixed effect. As any relationship between dose and female weight may not be a straight line, a quadratic term (dose squared) was included in the model to assess curvilinearity. *G. bimaculatus* cricket data were analysed using a two-way ANOVA to investigate the effects of sex and treatment. The criterion for significance in all analysed was $p<0.05$.

## 5.3 Results

### 5.3.1 Injecting dsRNA into *G. legneri*'s host *C. cephalonica*

I injected 5 µL of dsRNA solution, the same volume that I used previously described in Chapter 4, using a 1 µg/µL dsRNA solution of *FoxO*, *EGFR*, *Myoglianin*, *DNMT3*, *DsRed* and injection buffer as control that the buffer is not harmful to either the caterpillars or the wasps. 13 caterpillars were not injected with anything after being stung by a *G. legneri* wasp and the adults emerged were used as negative control (Table 5.13). 524 *G. legneri* wasps were used from 62 caterpillars (*Myoglianin* host n= 10, wasp n=73. *DsRed* host n= 8, wasp n=62. Injection buffer host n= 9, wasp n=78. *FoxO* host n= 7, wasp n=66. *EGFR* host n= 9, wasp n=75. *DNMT3* host n= 6, wasp n=51. Untreated host n= 13, wasp n=119).

*Table 5.13 Molarity of the dsRNA solutions used in the first G. legneri wasp experiment.*

| dsRNA | Size | Concentration | Molarity |
|---|---|---|---|
| *FoxO* | 325 bp | 1 µg/µL | 4.76 µM |
| *EGFR* | 551 bp | 1 µg/µL | 2.41 µM |
| *Myoglianin* | 319 bp | 1 µg/µL | 4.84 µM |
| *DNMT1* | 615 bp | 1 µg/µL | 4.09 µM |
| *DNMT3* | 578 bp | 1 µg/µL | 3.08 µM |

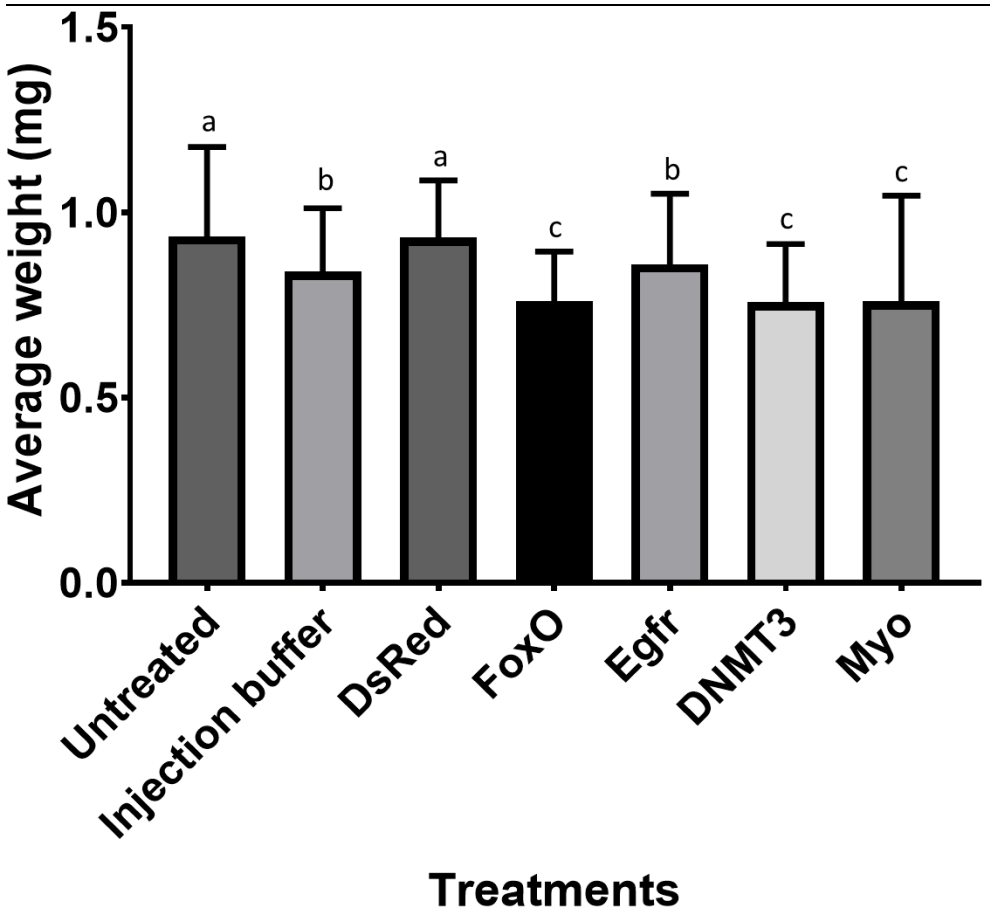


*Figure 5.4 Average adult female G. legneri wasp weight measurements for four 1 µg/µL RNAi treatments, FoxO, EGFR, Myoglianin and DNMT3; and three negative controls, 1 µg/µL DsRed , injection buffer and untreated hosts. Error bars represent standard error. The experiment ran for 8 days until adult wasps emerged from the cocoons. Untreated n=64 from 5 hosts, Injection buffer n=78 from 9 hosts, DsRed n=62 from 8 hosts, FoxO n=66 from 7 hosts, Egfr n=75 from 9 hosts, DNMT3 n=51 from 5 hosts, Myoglianin n=73 from 9 hosts. Treatments labelled with the same letter above the bar did not differ significantly from each other (p>0.05, but did differ (p<0.05)from those labelled with different letters, as established by progressive aggregation of treatment factor levels.*

The influence of host weight on *G. legneri* wasp weight was analysed using a generalised mixed linear module approach. Wasp weight was not significantly influenced by host weight ($F_{1,53.7}$ = 4.12, $p$=0.047). The effect of treatment was analysed by ANOVA and showed a significant difference between treatments ($F_{5,399}$ = 8.86, $p$<0.001). I performed a progressive aggregation of treatment factor levels to reach the minimal adequate model (Crawley, 1993): three groups could be formed until no more aggregation could be performed showing that the three groups were significantly different from each other ($F_{2,521}$ = 34.26, $p$<0.001). The three groups are identified by a single letter: *a* contains untreated *G. legneri* wasps and *DsRed* treatment; *b* includes *G. legneri* wasps from injection buffer and *EGFR* treatments; *c* comprises *DNMT3*, *FoxO* and *Myoglianin* treatments.

A second experiment was carried out using the same molarity for each dsRNA solution instead of the same concentration. I used 5 µL of 10 µM dsRNA solution for *FoxO*, *EGFR*, *Myoglianin*, *DNMT1*, *DNMT3* and *DsRed*. I also used untreated *G. legneri* wasps and *G.*

*legneri* wasps that had developed on caterpillars injected with only the injection buffer as control treatments. The concentration and molarity of the second batch was high enough to produce a 10 µM solution for every dsRNA (Table 5.10). 567 *G. legneri* wasps were used from 67 hosts (*Myoglianin* host n= 10, wasp n=73. *DsRed* host n= 7, wasp n=73. Injection buffer host n= 9, wasp n=78. *FoxO* host n= 8, wasp n=52. *EGFR* host n= 7, wasp n=59. *DNMT1* host n= 7, wasp n=52. *DNMT3* host n= 7, wasp n=43. Untreated host n= 13, wasp n=119). Results are shown in Figure 5.5. The influence of host weight on *G. legneri* wasp weight was analysed using a generalised mixed linear module approach and had no significant effect ($F_{1,58.2}$ = 1.26, $p$=0.267). The effect of treatment on wasp weight was, however, significant (ANOVA $F_{7,559}$ = 35.82, $p$<0.001). I performed a progressive aggregation of treatment factor levels to obtain the minimal adequate model (Crawley, 1993): treatment levels could be aggregated into four groups without causing a significant change in the variance ratio, but differences between these four groups were significant ($F_{3,562}$ = 81.97, $p$<0.001). The four groups are identified by a single letter: *a* is the untreated *G. legneri* wasps; *b* includes *G. legneri* wasps from injection buffer and *FoxO* treatments; *c* comprises *EGFR*, *DNMT1* and *DsRed*; and finally, *d* includes *G. legneri* wasps from *DNMT3* and *Myoglianin* (Figure 5.5).

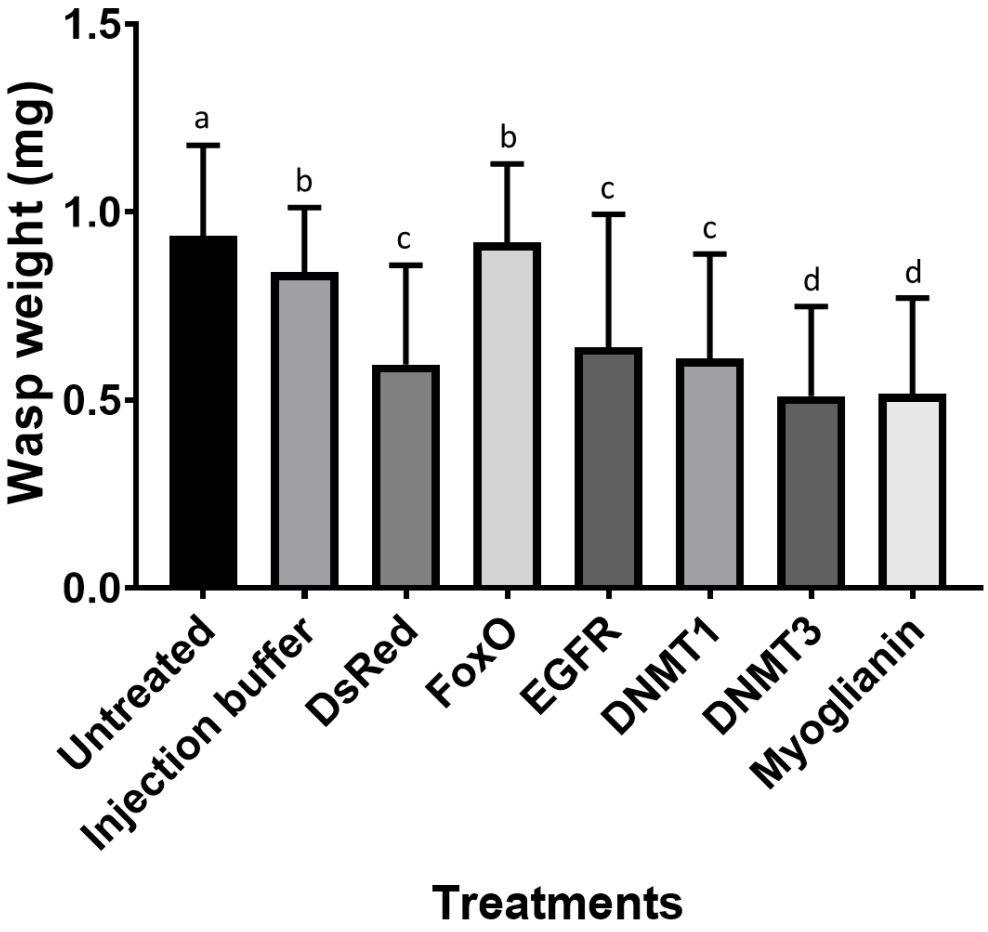



*Figure 5.5 Average adult female G. legneri wasp weight measurements for five 10 µM RNAi treatments, FoxO, EGFR, DNMT1, DNMT3 and Myoglianin; and three negative controls, 10 µM DsRed, injection buffer and no treatment. Error bars represent standard error. The experiment ran for 8 days until adult wasps emerged from the cocoons. Untreated n=64 from 5 hosts, Injection buffer n=78 from 9 hosts, DsRed n=90 from 9 hosts, FoxO n=52 from 8 hosts, Egfr n=59 from 7 hosts, DNMT1 n=52 from 7 hosts, DNMT3 n=43 from 7 hosts, Myoglianin n=73 from 7 hosts. The letters a, b, c, and d indicate the grouping obtained after successive aggregation of treatments factor levels, differences between these four groups were significant ($F_{3,562}$ = 81.97, p<0.001).*

### 5.3.2 Injecting dsRNA into *G. bimaculatus*

Four different treatments were injected in *G. bimaculatus* crickets: *FoxO*, *EGFR*, *DNMT1* and *DsRed*, each of them at 20 µM concentration (Table 5.11). The choice of molarity for the RNAi-probes used for the treatment for *G. bimaculatus* crickets was based on experiments described by Nakamura et al. (Nakamura et al., 2008b). There was also an untreated *G. bimaculatus* cricket (control) treatment.

As already mentioned in Chapter 4, *G. bimaculatus* cricket nymphs do not exhibit sexual dimorphism. Injections were thus performed on unsexed nymphs and only once these had developed to adulthood, had I been able to form two groups to compare treatments taking cricket sex into account. Therefore, the numbers of males and females were not balanced for each treatment type. A total of 54 *G. bimaculatus* crickets were analysed (females n=34, males n=20) using two-way ANOVA. The effects of treatment and the influence on sex were analysed. Cricket weight was not affected by treatment ($F_{4,44} = 0.91$, $p$=0.466),while sex affected weight significantly ($F_{1,44} = 55.61$, $p$<0.001), with females typically weighing more than males across all treatments (Figure 5.6). The interaction between sex and treatment did not produce significant effects ($F_{4,44} = 0.41$, $p$=0.801).

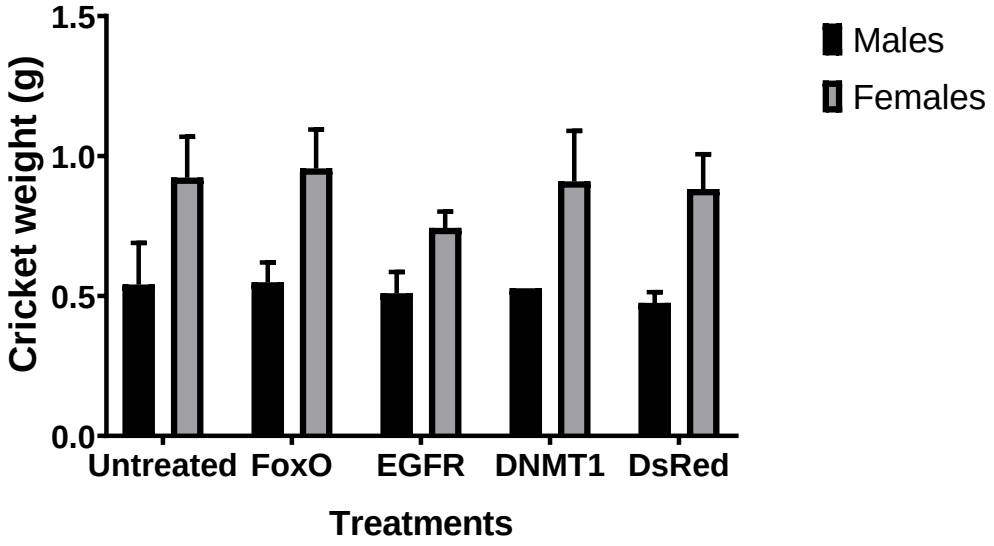



*Figure 5.6 Merged average adult female and male weight measurements for three 20 µM RNAi treatments, FoxO, EGFR and DNMT1; and two negative controls, DsRed and untreated, on G. bimaculatus. Grey columns represent females, patterned columns represent males. Error bars represent standard error. The experiment ran for 21 days, until nymphs moulted into adults. Untreated n=24 (16Females, 8Males), FoxO n=8 (4F, 4M), EGFR n=6 (2F, 4M), Dnmt1 n=8 (7F, 1M), DsRed n=8 (5F, 3M). Treatment had no significant effect on weight ($F_{4,44}$ = 0.91, p=0.466). Sex had a significant effect on weight ($F_{1,44}$ = 55.61, p<0.001). Their interaction was not significant (F4,44 = 0.41, p=0.801).*

## 5.4 Discussion

In this chapter I described work that attempted to interfere with the expression of specific proteins during development of the cricket *G. bimaculatus* and the parasitoid wasp *G. legneri* by injecting dsRNA into either their body cavity or their food source.

I used the cricket *G. bimaculatus* because it is one of the insects reared commercially at Monkfield Nutrition Ltd (Chapter 1) and because successful RNAi studies have already been carried out on this species (Dabour et al., 2011), providing us with some of the

primer sequences to produce dsRNA and, more importantly, confirming that RNA interference on *G. bimaculatus* crickets is a valid and effective method to increase their size. Based on that information I chose a set of genes to be interfered with, that included genes involved in body size control (*EGFR*), genes involved in DNA methylation (*DNMT1* and *DNMT3*) and genes involved in muscular development (*Myoglianin*). I also aimed to reproduce the work done by Dabour et al. on *FoxO* (Dabour et al., 2011) because in that study they achieved body-enlarging effects on *G. bimaculatus* cricket, and it was one of the phenotypes that I tried to produce in the previous chapters.

The genes chosen to be knocked down were selected to obtain a growth in size in the adult insects. This phenotype was chosen because it is one of the modifications that differentiate queen bees and worker bees. Being able to repeat even some of these modifications was one of the aims decided with Monkfield Nutrition Ltd. The company's interest in obtaining modifications similar to the queen bee is due to the fact that the queen bee shows increased fertility, lifespan, and weight compared to a worker. Knocking down the expression of the gene *InR1* led to a 12.5% increase in honey bees, likely a product of increased feeding behaviour (Leonard et al. 2020). Crickets that had RNA interference against the gene *FoxO* had their weight increased significantly to 113.4% of the weight of individuals in the control treatment. This variability in weight increase between the two species is likely due to the different target gene, but it could also be ascribed to the different efficacy of the RNAi treatment between insects.

I was expecting enlarging effects for *FoxO*, *Myoglianin*, *DNMT1* and *DNMT3*, while for *EGFR* I was expecting a reduction of weight. What I obtained is a wide variation in sizes for all treatments, but the mean for each one was always smaller than that of untreated

individuals (control). The results I obtained do not reflect the paper of Dabour et al. (2011) on RNA interference on *G. bimaculatus* crickets. There was no previous evaluation of RNA interference in *G. legneri*. The results do not show any of the expected phenotypes, whether this is due to an interference of little to no effect or to an unsuccessful one will need more experimentation.

### 5.4.1 Quality of the dsRNA probes

One of the reasons the experiment may have not worked is the quality of the dsRNA. To rule out this possibility I performed many checks during the preparation of every probe to ensure their quality. The degenerate primers were built based on homologous genes other insect species, using the most conserved portions of the genes. The PCR products were sequenced by Source BioSciences (Nottingham, UK) and the sequences were blasted against the NCBI database to confirm that they had similarity with the original insects' homologous genes. Knowing the sequence length in bases, I checked each dsRNA sequence in agarose gels and compared their size to a ladder (section 2.2.16). All these steps allowed me to be confident on the quality of the dsRNA used.

### 5.4.2 dsRNA doses

There is no established concentration on how much dsRNA is needed for the interference to be successful in insects and different studies reported different concentrations that were used: 1 μg/μL in *Nasonia vitripennis* (Lynch and Desplan, 2006), 5 μg/μL in *Nilaparvata lugens,* 20 μg/μL in *G. bimaculatus* (Dabour et al., 2011).

The experiments with *G. legneri* involved two different dsRNA concentrations: the first experiment had the same concentration of 1 μg/μL for all RNA solutions (Table 5.13).

However, this meant that the solutions had different molarities. Due to this and to the fact that I did not obtain any new phenotypes, I decided to normalise the molarity of the injected solutions to 20 µM. There are studies that support the notion that higher doses of dsRNA are lethal to individuals (Tomizawa and Noda, 2018) but it is also possible that at low concentrations the dsRNA could have no effects at all due to biochemical degradation (Wang et al., 2016). Liu et al. (2010) suggest that experimenters must find the appropriate volume and concentration on a gene-by-gene basis, as each dsRNA has a different efficiency and that this is preferred over high-volume injections or multiple injections because exceeding certain doses could lead to high mortality. In the current case, *G. legneri* wasps did not exhibit dose-dependent mortality, but further increases in dosage may be needed to generate such effects.

The experiment on *G. bimaculatus* was based on the study by Dabour et al. (Dabour et al., 2011), following the injection methodology, the molarity of the solutions injected, and the choice of one of the genes knocked down they used. Dabour et al. (2011) tested three different developmental stages: third instar, fifth instar and eighth instar nymphs. I, however, only used the fifth instar nymphs and increased the injection volume from 350 nL to 5 µL (due to the lack of a microinjector). All *G. bimaculatus* crickets survived the injection and developed to adulthood, so the volume injected was not a cause of death. If the dsRNA doses were too high, I would have expected to see negative effects on the treated *G. bimaculatus* crickets compared to the untreated, which was not the case.

The injection volume depends on the insect size, as well, with few studies carried out on the effects of a higher or lower volume. The choice to inject 5 µL of solution in *C.*

*cephalonica* derived from experiments presented in Chapter 4, where I empirically identified the best volume to inject into *G. legneri* wasp's host. The same volume was used for RNA interference in both *G. legneri* wasps and *G. bimaculatus* crickets.

### 5.4.3 *In vivo* dsRNA degradation

RNA interference in insects has variable results due to the *in vivo* degradation of dsRNA (Wang et al., 2016, Singh et al., 2017). dsRNA can be degraded by RNases expressed in either the insect's salivary secretions, by the gut if ingested (Allen and Walker, 2012, Christiaens et al., 2014) or by body fluids if injected (Singh et al., 2017). The lack of RNAi components may also play a role in the interference efficiency, as dsRNA could be not degraded to siRNA in the body of the insect (Joga et al., 2016).

In my experiments, *G. legneri* was fed dsRNA, via its host, while *G. bimaculatus* was injected with a dsRNA solution. One of the factors of the lack of positive results of these experiments could be ascribed to the fact that *G. legneri* wasp larvae could be able to degrade the dsRNA with salivary or midgut's nucleases. Injection, using a microinjector, into larval or pupal stage *G. legneri* wasps would be a good way to test this possibility. In the case of *G. bimaculatus* crickets, the injection of dsRNA was known to produce results (Dabour et al., 2011) so degradation in the haemolymph can be ruled out.

It is important that in future studies a series of controls should be performed after the RNAi experiments. These controls will focus on investigating the reduction of expression of the silenced genes using real time quantitative PCR (RT-qPCR) or by using western-blot if the antibodies are available. This way the activity of the interference can be measured, and proper actions can be taken, such as increasing the dsRNA doses or

modifying the injection techniques. The correct delivery of the dsRNA probes could also be investigated either *in vitro* or *in vivo*. Most of the current studies are based on *in vitro* techniques like ELISA, immunoblotting, dot blot, immunofluorescence, immunohistochemistry, and immunoprecipitation due to the use of dsRNA-specific antibodies (Schönborn et al., 1991). Recently *in vivo* dsRNA visualisation has been perfected using dsRNA-binding proteins , in particular the J2 mAb, fused to a fluorescent protein, when these proteins bind to a dsRNA, they become fluorescent (Cheng et al., 2015; Monsion et al., 2018). This technique has been used on live cell lines, which means that to be able to use it on *G. legneri* larvae, they would have to be killed in the process. But also, they could be dissected and fixed to be imaged, to gain better insight on the dispersal of the probes, learning whether the probes are able to perform a systemic interference or if they are stopped at the gut barrier. Monsion et al. (2018) produced stable transgenic *Nicotiana benthamiana* lines to better visualise the evolution of dsRNA *in vivo*. This technique is still far from being available to be used on any insect, as specific cell lines are challenging to produce.

A technique specific to investigate the reduction of methylation is called methylation sensitive amplified fragment length polymorphism (MS-AFLP) and is used to compare methylation patterns (Shaham et al., 2016). This would be done on control individuals and on treated ones to see if the knockdown of *DNMTs* creates a reduction in DNA methylation.

### 5.4.4 Insect developmental stage

The choice of the developmental stage of *G. bimaculatus* crickets and *G. legneri* wasps to use in the experiments was important: as the efficiency of RNA interference on the same target gene can differ depending on the different stages of the insect (Liu et al., 2010). Dabour et al.'s study (Dabour et al., 2011) provided guidance for the choice of *G. bimaculatus* cricket instar to be studied: the fifth instar of crickets is a good compromise between length of the experiment and contact with the dsRNA. For *G. legneri* wasps, being unable to inject directly into developing larvae without causing their death by mechanical disruption limited me to exposing the developing *G. legneri* wasp to dsRNA via larval feeding. Being able to inject into the pupae in future work may prove informative.

### 5.4.5 dsRNA delivery

Other dsRNA delivery methods could be used to assess the effectiveness of RNA interference treatment on *G. legneri* and *G. bimaculatus*. Zhang et al. (2010) used polymeric nanoparticles to cover the dsRNA probes to encapsulate them to reduce dsRNA degradation and to increase the cellular uptake, this way they were able to produce interference in mosquitoes. The nanoparticles were made of Chitosan, which deposits itself on the dsRNA due thanks to electrostatic forces between the amino groups in the chitosan and phosphate groups on the backbone of the nucleic acid. Chitosan polymers are stable, non-toxic, and easily biodegradable (Dass and Choong, 2008).

One of the reasons why the RNA interference did not work on *G. legneri* could be because the dsRNA probes had to pass through their digestive system and were blocked by it. Liposome vesicles were used to successfully deliver dsRNA fed to *Drosophila* compared to feeding non-encapsulated dsRNA (Whyard et al. 2009). This technique is already used to deliver drugs to remove the risk of degradation (Gregoriadis, 1977), liposomes are non-toxic and bio-degradable, making it a good choice to make dsRNA pass through the insects' gut.

dsRNA-producing bacteria have been used to perform RNA interference; the first example was achieved using *E. coli* that were fed to *Caenorhabditis elegans* (Kamath et al., 2003). A specially engineered *E. coli,* deficient in RNase, was used to produce the dsRNA probes to feed Colorado potato beetle *Leptinotarsa decemlineata*, once the beetles fed on the bacteria, RNA interference was obtained (Zhu et al., 2011). The symbiotic bacteria *Snodgrassella alvi* has been used in honey bees, to induce RNA interference immune response. Once inside the host, the bacteria produced double-stranded RNA to activate RNAi and repress host gene expression (Leonard et al. 2020). The use of bacteria is a highly effective way to perform RNA interference, but it relies on the ability of the host to either feed on or be the host of a symbiotic bacteria in their gut. It is unknown what bacteria *G legneri* or *G. bimaculatus* possess in their gut systems, or if the two species could feed on a diet containing RNAi inducing bacteria. Further work is needed to be able to determine whether this is a feasible interference system for the insects that were used in this project, but the previous studies are encouraging in this regard.

# Chapter 6 Discussion

In this thesis a variety of methods has been used to gain a better understanding of the mechanisms behind the different growth trajectories that worker and queen bees experience during their development. There is still very little understanding of the process and the genes involved: to date no theory has managed to explain how honey bees to influence the developmental destiny of larva via diet. Queen bees were reared through the addition of Royalactin to a royal jelly devoid of proteins that was fed to honey bee larvae (Kamakura, 2011); this suggested that a single component was able to trigger the modifications that produce a queen bee. Very similar results were obtained in Kucharski's study (Kucharski et al., 2008) by inhibiting the expression of the DNA methyltransferase 3. The study did not utilize bees directly; rather, the aim was to obtain this new knowledge using different species including commercially reared insects and also insects that are utilized in biological pest control, ideally obtaining phenotypes with increased body weight given that this is likely to enhance both commercial value and biocontrol performance. Based on the results of these two studies, this thesis followed a path that involved working on the epidermal growth factor receptor pathway using Royalactin, and then inhibiting the expression of DNA methyltransferases using inhibitors and through gene knockdown.

The aim of Chapter 3 was to produce a recombinant version of Royalactin using *Escherichia coli* and then purify it to add it to the diet of specific insects to test its ability to trigger queen-like modifications. Four different cell lines were used to express the protein (section 3.3.1), two of which were not able to produce it. The two that positively expressed the protein are cell lines engineered to express toxic proteins, which suggests

that Royalactin possesses some antibiotic properties. The experiments involved tuning of the expression to find the best expressing conditions: temperature, IPTG concentration, initial cell culture O.D. and expression time were independently analysed to achieve the highest yield (section 3.2.2).

Different purification trials were performed, but none of them produced the expected results as the protein was never completely purified (section 3.3.4). The recombinant protein expresses a HIS-tag which is used in the purification techniques to link to the affinity column. To ensure that the protein's tertiary structure was not hiding the HIS-tag, denaturing conditions were used to linearize the protein, but the protein was still washed away without binding to the column. More work is needed to tune the purification and to be able to use the recombinant protein in feeding trials on insects.

Due to the inability to purify Royalactin to feed insects, the focus was shifted on an alternative method to produce queen-like effects on honey bees with the intention of testing the same pathway on different insects. In Chapter 4, DNA methylation inhibitors were used to treat the wasp *Goniozus legneri* and the cricket *Gryllus bimaculatus*. The aim was to reproduce a study on honey bees where the silencing of DNA methyltransferases in larvae, led to the birth of queen bees. The inhibitor RG108 links to the DNA methyltransferase preventing it to bind to cytosines. RG108 did not produce any significant effects, even though its activity has been confirmed in insects (Cardoso-Junior et al., 2018) (sections 4.4.2 and 4.4.3). Different treatments were tested, from injecting the chemical to topical treatment as the absorption pathway can influence the outcome. No difference was found between treatments, which suggests that either RG108 was degraded before being able to produce any phenotype or that the chemical

needs to be injected in a more controlled form. To do so, a microinjector would need to be used; most of the studies in which chemicals are injected into insects employ them (Arakane et al., 2004; Suzuki et al., 2008, Yu et al., 2013),as the dosage is more controlled and the stability and smaller size of the needle assist with the precision of injection.

The other DNA methylation inhibitor used in Chapter 4 is 5-azacytidine, this inhibitor affects DNA methylation by inserting itself in the DNA in place of cytosines during DNA replication and by blocking the DNMT enzymes that normally bind to perform the methylation. The experiment shows a dose-dependent weight increasing effect on *G. legneri* (section 4.4.1). Since the demethylation occurs on the entire genome, it is impossible to pin point the genes that are involved in this effect. These results stimulated me to analyse the silencing of specific genes to better understand what genes are directly involved in the pathway leading to body enlarging effects.

In Chapter 5 RNA interference was used to study the silencing of genes involved in the development of insects. These experiments were designed to link the previous two chapters together and understand the influence of single genes on the development of insects. The genes that were investigated include EGFR, which is part of the pathway which Royalactin influences, and DNA methyltransferase genes, which were inhibited using 5-azacytidine and RG108 in Chapter 4. RNA interference was chosen because of its specificity towards single genes, as opposed to the more generalised approach of the previous two chapters. The aim of this chapter was to analyse the effects of the silencing genes involved in the pathways previously studied, with the addition of a gene involved in muscle development, which has not been silenced in insects before.

There was neither an increase nor a decrease in weight in the wasp *G. legneri* and in the cricket *G. bimaculatus*. Future work on the topic will include a dose response of the dsRNA probes and a series of experiments to analyse the activity of the knockdown using western blots or fluorescent probes to check the gene expression, or using RNA extraction, cDNA synthesis and RT-qPCR to check the RNA expression.

Overall, the hypothesis that Royalactin can influence body weight, longevity and fecundity of other insects than honey bees has not been yet confirmed. More work on refining Royalactin's purification is needed to verify the conclusions of Kamakura (2011) that one single component in royal jelly can lead to the development of fully formed queen bees, and that the same component can increase body weight in other insect species. Studying the silencing of genes involved in Royalactin's pathway is an elegant way to test which genes are responsible for each of the queen-like modifications. Unfortunately, the experiments did not produce any significantly different phenotypes, either in *G. legneri* or in *G. bimaculatus*.

The use of the DNA methylation inhibitor 5-azacytidine produced a significant dose-dependent increase in weight in the wasp *G. legneri*. Conversely, the DNA methylation inhibitor RG108 did not produce any significant modifications in either *G. bimaculatus* or *G. legneri*, even though more administration methodologies, including topical applications, were trialled. Hopefully, improving Royalactin's purification technique, DNMT treatment methodologies and finally dsRNA administration will yield better results that can be used to tie these three topics into one explanation of the functions of Royalactin and the best way to produce enlarged body phenotypes in insects.

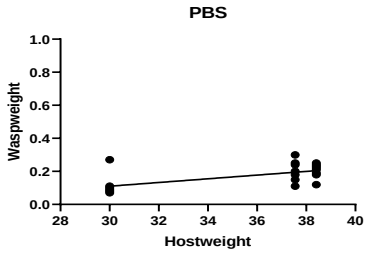
# Appendix A Host weight vs *G. legneri* wasp weight

The correlation between host weight and *G. legneri* wasp weight was analysed using the program Minitab. Pairwise Pearson correlation test was used on one variable at a time, averaging every *G. legneri* wasp weight per host, the test's r (correlation) and p values are reported under the graphs of Figure A.1. A correlation between all treatments (excluding negative controls) was performed and the results are shown in Table A.1. The correlation value ranges between 1 and -1, a value of 1 means that the relationship between the two variables is described by a linear equation, a value of -1 means that the relationship is described as a negative correlation. A value of 0 describes no linear correlation between the variables.

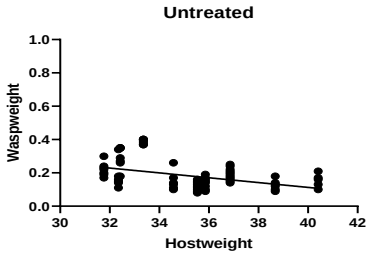
*Table A. 1 Pairwise Pearson correlations between host weight and G. legneri wasp weight.*

| Variable 1 | Variable 2 | Correlation | P-Value |
|---|---|---|---|
| Wasp weight | Host weight | 0.075 | 0.597 |

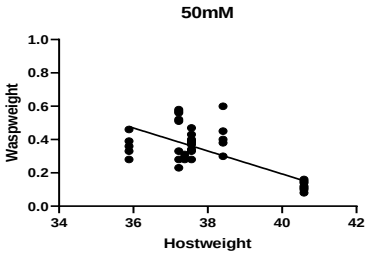
The general Pairwise Pearson test reports no significance between the two values.



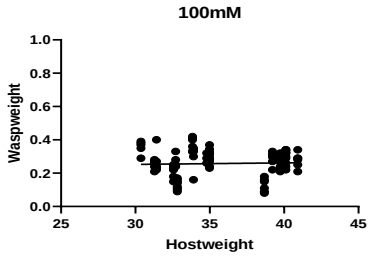

r=0.999
p=0.030



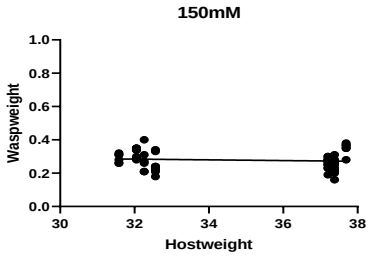

r=-0.501
p=0.140



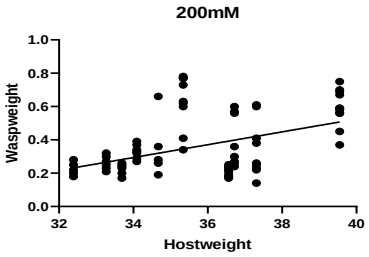

r=-0.701
p=0.121



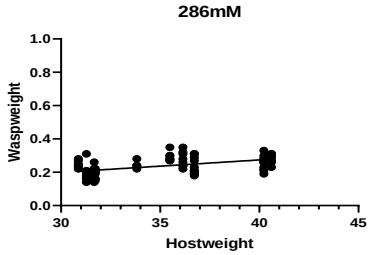

r=-0.104
p=0.713




r=-0.026
p=0.956




r=0.587
p=0.074




r=0.672
p=0.033

*Figure A. 1 Relationships between host weight and emerged wasp weight in mg. Host weight was recorded, then the host was paralysed by a wasp and then the treatment was injected. After eight days, once wasps had developed to adulthood, their weight was recorded. Separate graphs are shown for each experimental treatment: hosts were untreated or injected with PBS or 5-azacytidine at 50mM, 100mM, 150mM, 200mM or 286mM. r values of 0 indicate no correlation, +1 indicates a strong positive correlation, while -1 indicates a strong negative correlation.*

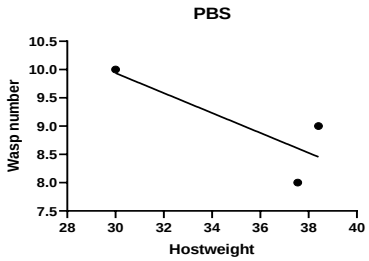
## Appendix B Host weight vs *G. legneri* wasp number

To investigate if there is a correlation between the weight of a host and the number of *G. legneri* wasps that spawns from it, a Pairwise Pearson correlation test was performed on the data. Figure B.1 shows the results of the Pairwise Pearson correlation test between host weight and *G. legneri* wasp number for each treatment that was injected into the host. A correlation between all treatments (negative controls excluded) was performed and the results are shown in Table B.1. The correlation value ranges between 1 and -1, a value of 1 means that the relationship between the two variables is described by a linear equation, a value of -1 means that the relationship is described as a negative correlation. A value of 0 describes no linear correlation between the variables.

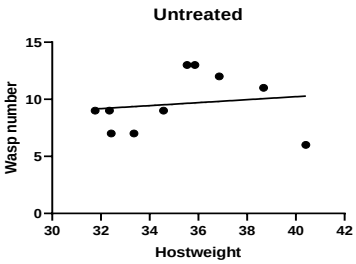
*Table B. 1 Pairwise Pearson correlations between host weight and emerged G. legneri wasp number.*

| Variable 1 | Variable 2 | Correlation | P-Value |
|---|---|---|---|
| Wasp number | Host weight | 0.195 | 0.142 |

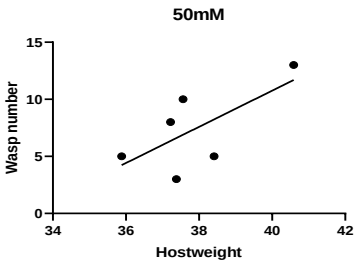
There is no significant correlation between the weight of the host and the number of wasps that developed.



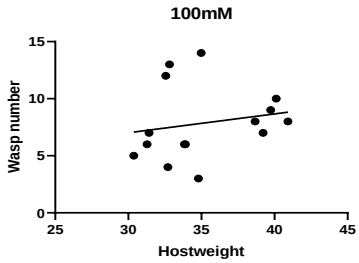

r=-0.861
*p*=0.393



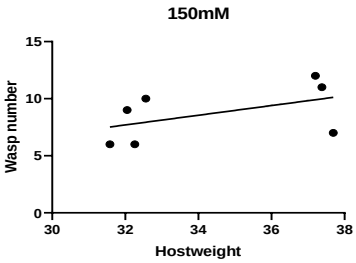

r=0.147
p=0.685




r=0.670
p=0.145




r=0.184
p=0.511

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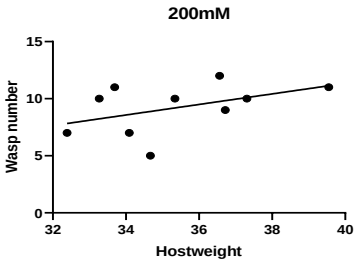
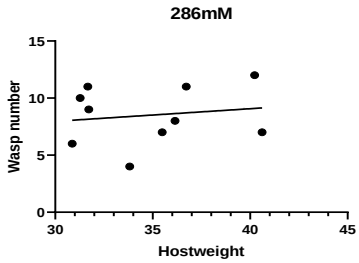
p=0.254




r=0.457
p=0.184




r=0.157
p=0.666

*Figure B. 1 Relationships between host weight and the number of adult wasps that developed. Host weight was recorded, then the host was paralysed by a wasp and then the treatment was injected. After eight days, the number of matured adult wasps was recorded. The graphs show data from different treatments separately: the hosts uninjected or were injected with PBS or with 5-azacytidine at 50mM, 100mM, 150mM, 200mM or 286mM). r values of 0 indicate no correlation, +1 indicates a strong positive correlation, while -1 indicates a strong negative correlation.*

# Appendix C Multiple sequence alignments

## C.1 FoxO

*Table C. 1 List of sequences aligned to find conserved regions for FoxO, and the names of the species examined.*

| Sequence name | Insect species |
|---|---|
| XM_016913181.1 | *Apis mellifera (Honey bee)* |
| NM_001316497.1 | *Drosophila melanogaster (Fruit fly)* |
| AB557980.2 | *Gryllus bimaculatus (Cricket)* |
| XM_008204819.2 | *Nasonia vitripennis (Parasitoid wasp)* |

```
XM_016913181.1 AM      GAAGAATTCCTCCCGACGGAATGCCTGGGGAAATCACAGTTATGCAGATCTCATTACTCA
NM_001316497.1 DM      GAAGAACTCATCGCGTCGCAATGCATGGGGAAATCTATCCTATGCGGATCTCATCACGCA
AB557980.2 GB          -------ACCACCCGGCGGAACGCGTGGGGTAACCTGTCGTACGCGGACCTCATCACGCC
XM_008204819.2 NV      AAAGAACTCGTCCCGCCGGAACGCTTGGGGTAACCTCAGCTACGCCGATCTCATCACCCA
                                *  * ** ** ** ** ***** ** *     ** ** ** ***** ** *

XM_016913181.1 AM      AGCCATTACCAGTGCTCCAGATGAACGGTTGACTTTGTCTCAGATCTACGAATGGATGAT
NM_001316497.1 DM      TGCCATTGGATCGGCCACCGACAAACGATTGACACTGAGTCAGATTTACGAGTGGATGGT
AB557980.2 GB          GGCAATCGCGTCGGCGCCCGAGAAGCGGCTCACCCTGTCGCAGATCTATGAGTGGATGGT
XM_008204819.2 NV      GGCCATCACCTCGGCGCCCGACAAGCGGCTAACCCTCTCGCAGATCTACGAGTGGATGGT
                         ** **       **  * **  * **  * **  *    ***** ** ** ****** *

XM_016913181.1 AM      GCAGAATATTCCATATTTCAGGGAGAAGGGAGAAAGCAACAGTAGCGCCGGATGGAAGAA
NM_001316497.1 DM      CCAGAATGTGCCATATTTCAAGGACAAGGGCGATTCGAATAGCAGTGCCGGATGGAAGAA
AB557980.2 GB          GCAGAACGTGCCCTACTTCGAAGACAAGGGCGACAGCAACAGCTCCGCCGGCTGGAAGAA
XM_008204819.2 NV      CCAGAACGTGCCCTACTTCAAGGACAAGGGCGACAGCAACAGCAGTGCCGGATGGAAGAA
                         *****  * ** ** ***   ** ***** **    ** **    ***** ********

XM_016913181.1 AM      CTCTATAAGGCACAACTTATCGTTACATAGCAGATTTATGCGAGTGCAGAACGAGGGTAC
NM_001316497.1 DM      CTCCATACGTCACAATCTGTCGCTGCACAACCGCTTTATGAGGGTCCAAAACGAGGGCAC
AB557980.2 GB          CTCGATCCGACATAACCTGTCGCTGCACAACCGCTTCATGCGCGTGCAGAACGAGGGCAC
XM_008204819.2 NV      CTCCATCAGACACAACCTCTCGCTGCACAACAGATTCATGCGGGTTCAGAACGAGGGTAC
                        *** **  * ** **  * *** * ** * * * ** *** * ** ** ******** **

XM_016913181.1 AM      CGGAAAAAGTTCCTGGTGGATGATCAATCGTGATGCGAAACCAGGGAAATCACGTAGAA-
NM_001316497.1 DM      CGGCAAGTCATCCTGGTGGATGCTCAACCCGGAGGCCAAGCCCGGCAAGTCTGTGCGCCG
AB557980.2 GB          GGGCAAGTCGTCGTGGTGGATGATCAACCCGGACGCGAAGCCGGGCAAGAGCGCGCGCCA
XM_008204819.2 NV      GGGAAAGAGCTCCTGGTGGATGATCAACCCCGACGCCAAGCCCGGAAAGTCAGCTAGGAG
                         ** **    ** ********* **** *  ** ** ** ** ** **        *

XM_016913181.1 AM      --GAGCAATAACCATGGAGACGAGCAAATTTGAGAAACGACGTGGTAGGGTAAGGAAGAA
NM_001316497.1 DM      CCGTGCCGCTTCCATGGAGACGTCCCGGTACGAGAAGCGGCGCGGCAGGGCCAAGAAGCG
AB557980.2 GB          CCGCGCCACCTCAATGGAGACTCCTAAATTCGAGAAG----------------------
XM_008204819.2 NV      AAGAGCCACGTCGATGGAGACGTCCAAGTTCGAGAAGCGTCGGGGCCGAGTGAAGAAGAA
                          * **     * ********       *  *****
```

*Figure C. 1 Multiple aligned sequences to find portions of conserved DNA. The highlighted area represents the sequence selected to create the degenerate primers for FoxO. The stars under the sequence signal the conserved bases in all aligned sequences. AM= Apis mellifera, DM= Drosophila melanogaster, GB=Gryllus bimaculatus, NV= Nasonia vitripennis.*

## C.2 EGFR

*Table C. 2 List of sequences aligned to find conserved regions for EGFR, and their respective organism names.*

| Sequence name | Organism |
|---|---|
| XM_006560026.2 | *Apis mellifera (Honey bee)* |
| NM_057410.4 | *Drosophila melanogaster (Fruit fly)* |

| | |
|---|---|
| AB300616.1 | *Gryllus bimaculatus (Cricket)* |
| XM_008215821.2 | *Nasonia vitripennis (Parasitoid wasp)* |

```
NM_057410.4 DM        ACCTCAGAGATCGGTACACGAACTGTACGTATGTGGATGGCAACCTGGAGCTGACCTGGC
AB300616.1 GB         ACCTGCGCGAGCGCTACACCAACTGCACCTACGTGGACGGCAACCTTGAGGTGACGTGGC
XM_006560026.2 AM     ATCTTCGAGACCGGTACACCAACTGTACTTACGTTGACGGCAATCTGGAGATCACATGGC
XM_008215821.2 NV     ACCTTCGCGACCGGTACACCAACTGCACGTATGTCGACGGCAACCTCGAGATCACCTGGC
                      * **  * ** ** ***** ***** ** ** ** ** ***** ** *** * ** ****

NM_057410.4 DM        TGCCCAACGAGAATTTGGACCTCAGCTTCCTGGACAACATACGGGAGGTCACCGGCTATA
AB300616.1 GB         TGGAGGACCCGCAGCTGGACCTGAGCTTCCTGCAGCACATCCGCGAGGTGACGGGCTACG
XM_006560026.2 AM     TCCAGAACGAGACATTCGACCTCAGTTTCCTCCAGTATATACGGGAGGTCACAGGTTACG
XM_008215821.2 NV     TGCAGGACGAGAAGTTCGACCTCAGCTTCCTGCAGTACATCCGCGAGGTCACCGGCTACG
                      *     **  *    * ***** ** *****  *  * ** ** ***** ** ** **

NM_057410.4 DM        TTCTGATCAGTCATGTGGACGTTAAGAAAGTGGTATTTCCCAAACTACAAATCATTCGCG
AB300616.1 GB         TGCTCATCTCGCACGTGAACGTGCCCCGCGTGGTGCTGCCGGCGCTGCAGATCATCCGCG
XM_006560026.2 AM     TGCTCATCAGCCACGTGGACGTCAAGAAGATCGTTTTGCCGCGATTGCAGATCATACGAG
XM_008215821.2 NV     TCCTCATCAGCCACGTCGACGTGCCCAAGATGGTCCTGCCGAGGCTGCAGATCATCCGCG
                      * ** ***   ** **  ****        * **  * **     * ** ***** ** *

NM_057410.4 DM        GACGCACGCTGTTCAGCTTATCCGTGGAGGAGGAGAAGTATGCCTTGTTCGTCACTTATT
AB300616.1 GB         GGCGAACGCTCTTCCGCATCGCGGTGCACGACGAGGACTTCGCGCTCATGATCCTGCTCT
XM_006560026.2 AM     GAAGAACACTGTTCAAGCTCACCATTCATGACATCGAGTTCGCCCTATTCGTCACGATGT
XM_008215821.2 NV     GCCGGACGCTCTTCAAGGTCAACATCGACGCGAACGAGTACGCGCTCTTCGTCACCATGT
                      *  * ** ** ***    *     *  * *      * *  **  *  *  **      *

NM_057410.4 DM        CCAAAATGTACACGCTGGAGATTCCCGATCTACGCGATGTCTTAAATGGCCAAGTGGGCT
AB300616.1 GB         CCAAGATGAACAGCCTCGAGATGCCGGCGCTCAGGGATATCCTTCAAGGAAACGTGGGTC
XM_006560026.2 AM     GTCAGATGCAGAACTTATCACGCGTGTCGTTGCCAGATATCCTCAACGGCAGCGTGGGCA
XM_008215821.2 NV     GCCAGATGTTTAACCTCGAGATGCCGGCGCTCAGGGACATCCTCAACGGCAGCGTGGGCA
                         * ***   *   *             *    **  ** *  * **    *****

NM_057410.4 DM        TCCACAACAACTACAATCTCTGCCACATGCGAACGATCCAGTGGTCGGAGATTGTATCCA
AB300616.1 GB         TATTGAATAACTACAACTTGTGCCACATTAGAACAATTAACTGGGATGACATCATCACTG
XM_006560026.2 AM     TGTACAACAACTACAACCTGTGCCACATCCGCACGATCAACTGGGAGGAAATAATCACAG
XM_008215821.2 NV     TCTTCGACAACTACAACCTCTGCCACGTCAAGTCGATCAAGTGGGACGAGATCATAACCG
                      *     * ********  * ****** *     * **  * ***   ** **  *  *

NM_057410.4 DM        ACGGCA---CGGATGCATACTACAACTACGACTTTACTGCTCCGGAGCGCGAGTGTCCCA
AB300616.1 GB         GACCGGGTGCTCATGTTGTACACGTGTACAACTTCACAATGCCAGAACGGGAATGTCCTC
XM_006560026.2 AM     GGCCGAGTGCAATGTACTCCTACGTGTACAATTTTACGTCTCCGGAACGCGCCTGTACAC
XM_008215821.2 NV     GGCAGGGTCGGAACTACAGCTTCGTCTACAACTTCTCGACGCCCGAGCGCGTCTGCCCGC
                                        *   *** * **  *    ** ** ** *  **  *

NM_057410.4 DM        AGTGCCACGAGAGCTGCACGCACGGATGTTGGGGCGAGGGTCCCAAGAATTGCCAGAAGT
AB300616.1 GB         CCTGTCATTCTTCCTGTGAAAATGGATGTTGGGGTGAAGGCCCTCATAACTGCCAGAAGT
XM_006560026.2 AM     CCTGCGATAAGAGCTGCGAGCAAGGCTGCTGGGGAGAGGGGCCTGAGAACTGTCAGAAGT
XM_008215821.2 NV     CCTGCGACAAGGAGTGCGAGGACGGCTGCTGGGGCGACGGCCCCAAGAACTGCCAGCGCT
                        **  *       **     * ** ** ***** ** ** **  * ** ** ***   *

NM_057410.4 DM        TCAGCAAGCTCACCTGCTCGCCACAGTGTGCCGGAGGTCGTTGCTATGGACCAAAGCCGC
AB300616.1 GB         TCTCAAAAATCAACTGCAGTCCACAGTGCCATCAGGGGCGGTGCTTTGGAACCAACCCAC
XM_006560026.2 AM     ATTCGAAGACAAACTGTTCGCCTCAGTGCTGGCAGGGCAGGTGCTTCGGGCCCAATCCGC
XM_008215821.2 NV     TCTCCAAGACCAAGTGCTCGCCCCAGTGCTGGCAGGGCCGCTGCTTCGGGCCCAACCCGC
                           **    *  **    ** *****       **  * ****  **  * ** ** *

NM_057410.4 DM        GGGAGTGTTGTCACCTCTTCTGCGCCGGAGGATGCACTGGTCCCACGCAAAAGGATTGCA
AB300616.1 GB         GCGAATGTTGCCACCTGTTCTGCGCTGGTGGCTGTCGTGGTCCTAAACAGAGCGACTGCT
XM_006560026.2 AM     GGGAGTGTTGCCATCTTTTTTGCGCCGGTGGCTGCACAGGCCCGAAACAGAGCGACTGCA
XM_008215821.2 NV     GCGAGTGCTGCCACCTGTTCTGCGCGGGCGGTTGCACCGGGCCCAAGCAGAGCGACTGCC
                      * ** ** ** ** ** ** ***** ** ** **    ** ** *  ** *  ** ***
```

*Figure C. 2 Multiple aligned sequences to find portions of conserved DNA. The highlighted area represents the sequence selected to create the degenerate primers for EGFR. The stars under the sequence signal the conserved bases in all aligned sequences. AM= Apis mellifera, DM= Drosophila melanogaster, GB=Gryllus bimaculatus, NV= Nasonia vitripennis.*

## C.3 Myoglianin

*Table C. 3 List of sequences aligned to find conserved regions for Myoglianin, and their respective organism names.*

| Sequence name | Insect species |
|---|---|
| LC128665.1 | Gryllus bimaculatus |
| XM_016916400.1 | *Gryllus bimaculatus (Cricket)* |
| XM_006621203.1 | *Apis mellifera (Honey bee)* |
| XM_016982834.1 | *Apis dorsata (Giant honey bee)* |
| XM_015737513.1 | *Nasonia vitripennis (Parasitoid wasp)* |
| XM_015578631.1 | *Cephus cinctus (Wheat stem sawfly)* |
| XM_003402344.3 | *Dufourea novaeangliae (Shortface bee)* |
| XM_012390380.1 | *Bombus terrestris (Bumble bee)* |
| XM_012284026.1 | *Bombus impatiens (Bumble bee)* |
| XM_011259657.2 | *Megachile rotundata (Alfalfa leafcutting bee)* |
| XM_011063332.1 | *Camponotus floridanus (Carpenter ant)* |
| XM_012204970.1 | *Acromyrmex echinatior (Leafcutter ant)* |
| XM_011638852.1 | *Atta cephalotes (Leafcutter ant)* |
| XM_011174553.1 | *Pogonomyrmex barbatus (Harvester ant)* |

```
LC128665.1          ACTAAGCGCACCATCGGTCTGAATTGCGACGAATCGTCGGACGAGACGCGCTGCTGCCGC
XM_016916400.1      ATCAAGAGAAACGTAGGACTTAATTGCGACGAAGCCAGCCAAGAGACCAGATGTTGCCGA
XM_006621203.1      ATCAAGAGAAACGTAGGACTTAATTGCGACGAAGCCAGCCAAGAGACCAGATGTTGTCGA
XM_016982834.1      GTCAAGAGGAACGTCGGCCTCAACTGCGACGAGGCTAGTCAGGAGACCAGGTGCTGTCGC
XM_015737513.1      ATCAAGAGGAACGTTGGTCTGAACTGCGATGACTCGAGTCAGGAGACACGGTGCTGCCGC
XM_015578631.1      ATCAAAAGAAACATAGGACTTAATTGCGACGAGGCCAGCCAGGAGACCAGGTGCTGTCGA
XM_003402344.3      ATCAAGAGAAACGTGGGACTTAATTGCGACGAAGCTAGCCAGGAGACCAGATGTTGTCGA
XM_012390380.1      ATCAAGAGAAACGTGGGACTTAATTGCGACGAAGCTAGCCAGGAGACCAGATGTTGTCGA
XM_012284026.1      ATCAAGAGGAACGTAGGACTCAATTGCGACGAGGCCAGCCAAGAGACCAGGTGCTGTCGC
XM_011259657.2      ATCAAGAGAAACGTGGGACTCAATTGCGACGAGGCTAGTCAGGAGACTAGGTGTTGCCGG
XM_011063332.1      ATTAAGAGGAACGTGGGACTCAATTGCGATGAGGCCAGTCAGGAGACTAGATGTTGCCGG
XM_012204970.1      ATTAAGAGGAACGTGGGACTCAATTGCGATGAGGCTAGCCAGGAGACTAGATGTTGCCGG
XM_011638852.1      ATCAAGAGGAACGTGGGACTGAATTGCGACGAGGCCAGCCAGGAGACCAGATGTTGCCGG
XM_011174553.1      ATCAAGAGGAACGTGGGACTCAACTGCGACGAGGCCAGCCAAGAGACCAGGTGTTGTCGG
                       **  * * * * ** ** ** ***** **  *     * *****  * ** ** **

LC128665.1          TATCCTCTCACTGTCGACTTCGAAGAATTCGGCTGGGACTGGATCATCGCTCCCAAAAAA
XM_016916400.1      TACAAGCTCACCGTCGATTTTGAGAAATTTGGCTGGGATTGGATAATCGCGCCCAAGAAG
XM_006621203.1      TACAAACTCACCGTCGATTTTGAGAAATTTGGCTGGGATTGGATAATCGCGCCCAAGAAG
XM_016982834.1      TACAAACTCACCGTCGACTTTGAGAAGTTCGGATGGGACTGGATCATCGCGCCGAAAAAG
XM_015737513.1      TACAAGCTCACCGTGGACTTCGAAAAGTTCGGCTGGGATTGGATCATTGCGCCTAAAAAG
XM_015578631.1      TACAAGCTCACCGTCGACTTCGAGAAGTTCGGCTGGGACTGGATAATCGCGCCCAAAAAG
XM_003402344.3      TACAAGCTCACCGTCGACTTTGAGAAGTTTGGGTGGGATTGGATAATCGCTCCCAAGAAG
XM_012390380.1      TACAAGCTCACCGTCGACTTTGAGAAGTTTGGGTGGGATTGGATAATCGCTCCCAAGAAG
XM_012284026.1      TACAAGCTCACTGTGGACTTTGAGAAGTTTGGATGGGATTGGATCATCGCACCCAAGAAG
XM_011259657.2      TATAAGCTAACAGTGGACTTTGAAAAATTTGGCTGGGATTGGATAATCGCACCGAAAAAA
XM_011063332.1      TACAAACTTACGGTAGACTTCGAGAAGTTTGGCTGGGATTGGATAATCGCACCCAAAAAA
XM_012204970.1      TACAAACTCACGGTAGACTTCGAGAAGTTTGGTTGGGATTGGATAATCGCACCCAAAAAA
XM_011638852.1      TACAAGCTCACGGTAGACTTCGAGAAGTTCGGCTGGGATTGGATAATCGCACCCAAGAAA
XM_011174553.1      TATAAGCTCACGGTGGACTTTGAGAAGTTCGGCTGGGATTGGATAATCGCACCCAAAAAA
                    **    ** ** ** ** ** **  * ** ** ***** ***** ** ** ** ** **

LC128665.1          TACGAGGCCAACTACTGTTCGGGAGAATGCCCTTACGTATTCCTTCAGAAGTACCCTCAC
XM_016916400.1      TACGATGCCAATTATTGTTCGGGCGATTGTCCGATGGCCTTTCTACCGGCATATCCAAAC
XM_006621203.1      TACGATGCCAATTATTGTTCGGGCGATTGTCCGATGGCCTTTCTACCGGCATATCCAAAC
XM_016982834.1      TACGACGCGAATTACTGCTCAGGAGACTGTCCAATGGCGTTTCTTCCAGCCTACCCAAAC
XM_015737513.1      TACGACGCGAATTACTGTTCAGGCGACTGTCCAATGGCCTTTCTCCCAGCGTATCCGAAC
XM_015578631.1      TACGACGCCAATTACTGTTCGGGCGACTGTCCGATGGCGTTTCTCCAGGCCTATCCGAAC
XM_003402344.3      TACGATGCCAATTACTGCTCGGGCGACTGTCCGATGGCCTTCCTGCCAGCATATCCAAAC
XM_012390380.1      TACGATGCCAATTACTGCTCGGGCGACTGTCCGATGGCCTTCCTGCCAGCATATCCGAAC
XM_012284026.1      TACGACGCCAATTACTGTTCGGGCGATTGTCCGATGGCCTTTCTGCCAGCGTATCCAAAC
XM_011259657.2      TACGATGCCAATTACTGCTCGGGTGACTGTCCGATGGCTTTTCTTCCGGCCTATCCCAAT
XM_011063332.1      TATGATGCCAATTACTGTTCGGGTGACTGTCCAATGGCTTTTCTTCCGGCTTATCCGAAC
XM_012204970.1      TATGATGCCAATTACTGTTCGGGTGACTGTCCAATGGCTTTTCTTCCGGCTTATCCGAAC
XM_011638852.1      TACGATGCCAATTACTGTTCGGGTGATTGTCCGATGGCTTTCCTTCCGGCCTATCCAAAC
XM_011174553.1      TACGATGCCAATTACTGTTCGGGCGACTGTCCGATGGCTTTCCTTCCAGCCTATCCGAAC
                    ** ** ** ** ** ** ** ** ** ** **    *  ** ** *     ** **  *
```

*Figure C. 3 Multiple aligned sequences to find portions of conserved DNA. The highlighted area represents the sequence selected to create the degenerate primers for Myoglianin. The stars under the sequence signal the conserved bases in all aligned sequences.*

```
LC128665.1          ACGCACATCGTGCACCTGGCCCACCCCACGGGCACTGTGGGGCCATGCTGTGCGCCGCGC
XM_016916400.1      ACTCACATCGTCAGCCTGGCGGAGCCGCCCAACAACTCTGGTCCATGTTGCGCCCCGAGA
XM_006621203.1      ACTCACATCGTCAGCCTGGCGGAACCGCCTAACAACTCTGGTCCATGTTGCGCCCCGAGA
XM_016982834.1      ACACACATAGTGAGCTTGGCGGAGCCGCCGAACAACACGGGGCCCTGTTGTGCCCCGCGG
XM_015737513.1      ACGCACATAGTCTCGCTGGCGGAACCGCCGAATAACACGGGTCCCTGTTGTGCGCCCCGG
XM_015578631.1      ACCCACATCGTCAGCCTGGCGTTACCGCCGAACAACACCGGACCGTGTTGCGCACCGAGG
XM_003402344.3      ACCCACATCGTCAGCCTGGCGGAACCGCCTAACAACTCTGGTCCATGTTGCGCCCCGAGG
XM_012390380.1      ACCCACATCGTCAGCCTGGCGGAACCGCCTAACAACTCTGGTCCATGTTGCGCCCCGAGG
XM_012284026.1      ACCCACATCGTCAGCCTGGCGGAGCCACCGAACAACTCTGGTCCGTGCTGCGCCCCGAGG
XM_011259657.2      ACGCATATCGTCAGCCTAGCAGAACCGCCGAATAATACTGGACCATGCTGCGCGCCTAGA
XM_011063332.1      ACGCACATCGTCAGCTTGGCGGAACCGCCGAACAACACCGGACCATGTTGCGCACCTAGG
XM_012204970.1      ACGCATATCGTCAGCTTGGCGGAACCGCCGAACAACACCGGACCATGTTGCGCACCTAGG
XM_011638852.1      ACGCACATCGTCAGCCTAGCGGAACCACCGAACAACACCGGGCCATGCTGCGCCCCTAGG
XM_011174553.1      ACGCATATCGTCAGCCTGGCGGAACCGCCGAACAATACCGGGCCGTGCTGCGCACCTAGG
                    ** ** ** **     * **    **  *    *     ** ** ** ** ** **  *

LC128665.1          AAAATGTCGGCCATCTCCATGCTCTACTTCGACAACGAATTCAACATCATCTACGGCTTA
XM_016916400.1      AAACTCTCCGAGATCACGATGTTGTACTTCGATAACGAGTATCAAATCGTGTTTTCGCGA
XM_006621203.1      AAACTCTCCGAGATCACGATGTTGTACTTCGATAACGAGTATCAAATCGTGTTTTCGCGA
XM_016982834.1      AAGCTCTCGGAGATCACGATGCTGTACTTCGACAACGAGTACCAGATCGTCTTCTCGCGG
XM_015737513.1      AAGTTATCCGAGATCACGATGCTCTACTTCGACAACGAGTATCAGATAGTCTTCTCGCGA
XM_015578631.1      AAACTCTCCGAGATCACGATACTGTACTTCGACAACGAGTACCAGATCGTGTTCTCACGG
XM_003402344.3      AAACTCTCCGAGATCACGATGCTGTACTTCGATAACGAGTATCAAATCGTGTTCTCGCGA
XM_012390380.1      AAACTCTCCGAGATCACGATGCTGTACTTCGATAACGAGTATCAAATCGTGTTCTCGCGA
XM_012284026.1      AAACTGTCGGAGATCACGATGCTGTACTTCGACAACGAGTATCAGATCGTCTTCTCGCGG
XM_011259657.2      AAATTGTCCGAGATCACGATGCTGTACTTCGACAATGAGTATCAGATCGTGTTCTCGCGA
XM_011063332.1      AAGCTGTCCGAAATCACAATGTTATATTTTGACAATGAATATCAGATCGTGTTCTCGCGG
XM_012204970.1      AAGCTATCGGAAATCACAATGTTATATTTTGACAATGAATATCAGATCGTGTTCTCGCGG
XM_011638852.1      AAGCTGTCCGAGATCACGATGTTGTATTTTGACAACGAGTACCAGATCGTGTTCTCGCGA
XM_011174553.1      AAGCTGTCCGAAATCACAATGTTGTATTTTGACAACGAGTACCAGATCGTGTTCTCGCGG
                    **  * ** *  *** * **  * ** ** ** ** ** *   * **  * *

LC128665.1          CTCCCCGGCATGGTTGTCGATAGATGCGGTTGTTCGTAA----------------------
XM_016916400.1      TTACCAGGCATGGTCGTCGAGAGATGTGGATGCTCATAGTCTACCTTTATAGCTCGAGAG
XM_006621203.1      TTACCAGGCATGGTCGTCGAGAGATGTGGATGCTCATAGTCTACCTTTATAGCTCGAGAG
XM_016982834.1      CTGCCCGGCATGGTCGTCGAGCGCTGCGGCTGCTCATAGTCCCATAGCGAGGATGACGAA
XM_015737513.1      CTTCCGGGTATGGTGGTGGAGCGTTGCGGATGCTCATAGTCAATCGGTCAGGATCATCGT
XM_015578631.1      CTGCCGGGTATGGTCGTCGAGAGATGCGGATGCACATAG----------------------
XM_003402344.3      TTACCAGGCATGGTCGTCGAGAAATGTGGATGCTCATAGCTCGAGAATTATAGCTCGAGA
XM_012390380.1      TTACCAGGCATGGTCGTCGAGAAATGTGGATGCTCATAGCTCGAGAATTATAGCTCGAGA
XM_012284026.1      TTGCCGGGCATGGTCGTCGAGAGATGCGGATGCTCATAGTCCACCTTTCGGTTCGAGAAT
XM_011259657.2      CTACCGGGCATGGTAGTCGAGAAGTGTGGCTGCTCATAGTCCACCTACGAGTCAGACGGG
XM_011063332.1      TTGCCGGGCATGATCGTCGAGAAATGCGGGTGTTCATAGTCCACCTTCGTTTCTGACAGG
XM_012204970.1      TTGCCGGGCATGGTCGTCGAGAAATGCGGGTGTTCATAG----------------------
XM_011638852.1      TTGCCGGGCATGGTCGTCGAGAAATGCGGGTGCTCATAGTCCACCTTCGTTTCCGACGGG
XM_011174553.1      TTGCCGGGCATGGTCGTCGAGAAATGCGGGTGCTCATAGTCCACCTTCGTTTCTGGCAGG
                     * ** ** *** * ** **    ** ** **  * **
```

*Figure C. 4 Continued. Multiple aligned sequences to find portions of conserved DNA. The highlighted area represents the sequence selected to create the degenerate primers for Myoglianin. The stars under the sequence signal the conserved bases in all aligned sequences.*

## C.4 Dnmt3

*Table C. 4 List of sequences aligned to find conserved regions for Dnmt3, and their respective organism names.*

| Sequence name | Organism |
|---|---|
| XM_008206224.2 | *Nasonia vitripennis (Parasitoid wasp)* |
| XM_011174635.1 | *Solenopsis invicta (Fire ant)* |
| XM_011271310.2 | *Camponotus floridanus (Carpenter ant)* |
| XM_012199554.1 | *Atta cephalotes (Leafcutter ant)* |
| XM_011069215.1 | *Acromyrmex echinatior (Leafcutter ant)* |
| XM_015738858.1 | *Cephus cinctus (Wheat stem sawfly)* |
| XM_015579479.1 | *Dufourea novaeangliae (Shortface bee)* |
| XM_012281029.1 | *Megachile rotundata (Alfalfa leafcutting bee)* |
| XM_012386439.1 | *Bombus impatiens (Bumble bee)* |
| NM_001353392.1 | *Bombus terrestris (Bumble bee)* |
| NM_001190421.1 | *Apis mellifera (Honey bee)* |
| XM_006618195.1 | *Apis dorsata (Giant honey bee)* |

```
XM_008206224.2      ATGCAAGGATATAGTTGGTGGCCATGTGTTATCATGGATTATCAACATCTTAATCGTAAG
XM_011174635.1      ATCCCCGGACACTGTTGGTGGCCCGCGATGATTGTTGATTATCGTGATTGCTGTTTGAAA
XM_011271310.2      ATCGCTGGACATTGTTGGTGGCCCGCAATGATTATCGATTATCGCGACTGCTGCTTGAAG
XM_012199554.1      ATCCCTGGACATTGCTGGTGGCCCGCAATGATTATTGATTATCGCGACTGCTGTTTAAAA
XM_011069215.1      ATCCCTGGACATTGCTGGTGGCCCGCAATGATTATTGATTATCGTGACTGTTGTTTAAAA
XM_015738858.1      ATACCCGGGCATGCATGGTGGCCAGCAATGATAGTAGATCATCGGGATGCTGGTATGGAT
XM_015579479.1      ATCGCCGGGCACAATTGGTGGCCAGCGATGATTATCGATTACCGGGACTGTTGCATGCGT
XM_012281029.1      ATCTCGGGGCACAATTGGTGGCCAGCGATGATCATCGATTACCGCGACTGTTGCATGCGT
XM_012386439.1      ATCGCCGGACACAATTGGTGGCCAGCGATGATTATCGATTATCGTGACTGTTGCATGCGT
NM_001353392.1      ATCGCCGGACACAATTGGTGGCCAGCGATGATTATCGATTATCGTGACTGTTGCATGCGT
NM_001190421.1      ATTGCCGGGCACAATTGGTGGCCAGCGATGATTATCGATTATCGCGACTGTTGCATGCGA
XM_006618195.1      ATTGCCGGGCATAATTGGTGGCCAGCGATGATTATCGATTATCGCGATTGTTGCATGCGA
                    **    **  *    ********     * **  * *** * *   *

XM_008206224.2      CAGCCACATGTGGCACATCAATGGGTCATGTGGTATGGTGATTACAAGTATTCTCAAGTT
XM_011174635.1      GAACCGATTTTTGGTTGTCAATGGATTATGTGGTATGGAGATTATCAAGTTTCGGAGGTA
XM_011271310.2      GAACCGAGTTTTGGTTGTCAATGGATTATGTGGTACGGCGATTACAAAGTTTCAGAGGTA
XM_012199554.1      GAACCGAGTTTTGGTTGTCAATGGATTATGTGGTATGGGGATTATCAAGTTTCGGAGGTG
XM_011069215.1      GAACCGAGTTTTGGCTGTCAATGGATTATGTGGTATGGGGATTATCAAGTTTCGGAGGTG
XM_015738858.1      GAACCTAATTTCGGCTGCCAGTGGATCATCTGGTACGGCGACTACAAATTGTCTCAGGTG
XM_015579479.1      GAGCCGAGCTTCGGTTGCCAATGGATCATGTGGTACGGCGACTACAAGCTTTCAGAGATC
XM_012281029.1      GAGCCAAGCTTTGGTTGTCAGTGGATCATGTGGTACGGCGACTACCAACTTTCGGAGGTT
XM_012386439.1      GAACCAAGCTTTGGTTGCCAGTGGATTATGTGGTACGGTGATTACCAGCTCTCAGAGGTG
NM_001353392.1      GAACCAAGCTTTGGTTGCCAGTGGATTATGTGGTACGGTGATTACCAGCTCTCAGAGGTG
NM_001190421.1      GAACCGACGTTCGGTTGCCAATGGATCATGTGGTACGGCGACTACAAACTGTCGGAGGTG
XM_006618195.1      GAACCAACGTTCGGTTGCCAATGGATTATGTGGTACGGTGACTACAAACTGTCAGAGGTG
                     * **     * *     ** *** * ** ***** ** ** **  *    **  *  *

XM_008206224.2      CAGTATCGACAGATTCTTACTTTTCCAACAGGAATGGACAGAATGGAATCTAAAATTACC
XM_011174635.1      CGTCATCTCGAATTTCTGAAATTTCACAAAGGAATAGAAAAAATGCGCGAATATATTCAA
XM_011271310.2      CGTCATTTGGAATTTTTGAAATTTTACAAGGGACTGGAGAAGATGCGCGACTACATTCAA
XM_012199554.1      CGTCATCTCGAATTTTTGAAATTTCATAAAGGAATAGAAAAGATGCGAGAGTACATTCAG
XM_011069215.1      CGTCATCTCGAATTTTTGAAATTTCACAAAGGAATAGAAAAGATGCGAGAGTACATTCAG
XM_015738858.1      AATCATCGAGATTTTATGGCATTCGACTTGGGCTTCGAAAAAGTCCGCGAGTACACGGTG
XM_015579479.1      CATCATCAGTCGTTTTTGAGATTCGACAAGGGTGTCGAAAAAATGCGGGACTACATAAAC
XM_012281029.1      CATCACCAGCTGTTTCTAAGATTCGACAAAGGAATCGAGAGGATGCAAGACTACGTGAAA
XM_012386439.1      CACCATCAGTTGTTCTTGAGATTCGACAAGGGCATGGAAAAAATGCGCAACTACATAAAT
NM_001353392.1      CACCATCAGTTGTTCATGAGATTCGACAAGGGCATGGAAAAAATGCGCGACTACATAAAT
NM_001190421.1      CATCATCAATTGTTCTTGAGGTTCGACAAGGGGATGGAGAAAATGCGCGACTACACGAGC
XM_006618195.1      CATCACCAATTGTTTTTGAGATTCGACAAGGGAATGGAAAAAATGCGGGAATACACGAAC
                        *        *  *    **       **  * ** *   *        *

XM_008206224.2      GCAACAAAAGATGAGCTTTTTTGCAAGGCAGTTCTTCAAGCTGCTAAGGATTATTGTGAT
XM_011174635.1      AACAGTAATAGACAGCAGTATCTCGAAGGCGTGCTTCAGGCTTCCAAGGATTATTGCTCA
XM_011271310.2      AATACAGTTAAGCAGTGTTATCTCGACGGCGTGCTTCAAGCTTCGAAGGATTATTGTTCG
XM_012199554.1      AATACAAACAGACTACCATATCTTGAAGGCGTACTTCAAGCCTCCAAGGATTATTGCTCA
XM_011069215.1      AATACAAACAGACTACCATATCTTGAAGGTGTACTTCAAGCTTCCAAGGATTATTGCTCA
XM_015738858.1      CAGGCCAAGAGAGTCTCCTATGTAGCCGGCGTTCTCGATGCGTCAAAGGACTACTGCTCG
XM_015579479.1      AACACCAAGAAGCACACGTTCCTCGTCGGCGTTCTGCAAGCTTCGAAGGATTATTGCTTT
XM_012281029.1      AACACCAAGAAGCACGTCTATCTGGTCGGTGTCCTCCAAGCTGCGAAGGATTATTGTTCT
XM_012386439.1      AATACGAAAAAGCACATATACCTCGTTGGAGTTCTCCAAGCCTCCAAGGATTATTGCTCT
NM_001353392.1      AATACGAAAAAGCACATATACCTCTTTGGGGTTCTCCAAGCCTCCAAGGATTATTGCTCT
NM_001190421.1      AACACGAAGAAGCATATCTACCTCGTAGGAGTTCTCCAAGCCTCCAAGGATTATTGCTCC
XM_006618195.1      AACACGAAAAAACATATTTATCTCGTCGGTGTTCTTCAAGCCTCGAAGGACTATTGCTCC
                                    *        *  ** **  * **  * ***** ** **

XM_008206224.2      AAGTTGGGTTACTTAACTGAGCCATGGAAAATAAAGGATGTAATTCACCTATTTTATAAA
XM_011174635.1      CGTTTAGGATGCGACACGGATAACTGGACTTTAGATAATGTTTTTGAATATTTTTCGAAT
XM_011271310.2      CGTTTAGGATGCTCCACGGATAACTGGACTTTAGATAATGTGTTTGAATATTTTTCGAAT
XM_012199554.1      CGATTAGGATGCGACACGGATAACTGGACTTTAGATAATGTCTTTGAATATTTTTCAAAT
XM_011069215.1      CGGTTAGGATGCGACACGGATAACTGGACTTTAGATAATGTCTTTGAATATTTTTCAAAT
XM_015738858.1      CGACTTGGTTACAAGACTGACCACTGGAAGTTGACCGATGTACTGGGATGGTTCTCTCGA
XM_015579479.1      CGTATGGGATGCGAAACCGATAATTGGACGTTGGCGAACGTGTTCAAATATTTCTCGAAA
XM_012281029.1      CGTTTCGGATACGAAACCGAAAGTTGGACATTGGCCGACGTGTTCGAATACTTCTCGAAA
XM_012386439.1      CGTCTGGGATTCGAAACCAAGGACTGGACTGTAAACGACGCGTTGAGATATTTCGCAAGG
NM_001353392.1      CGTCTGGGATTCAAAACCGAGAACTGGACTGTAAACGACGCGTTGAAATATTTCGCAAGG
NM_001190421.1      CGTCTAGGATTCGATACCTCCAACTGGACTTTGGACGACGCTTTCGAATATTTTTCCAAG
XM_006618195.1      CGCCTAGGATTCGAAACCGTCAACTGGACTTTGGACGACGCGTTCGAATATTTTTCCAAA
```

*Figure C. 5 Multiple aligned sequences to find portions of conserved DNA. The highlighted area represents the sequence selected to create the degenerate primers for Dnmt3. The stars under the sequence signal the conserved bases in all aligned sequences.*

```
XM_008206224.2      TCCAAAGATATATACAAACTAAAAAATGCAGAGT----------------T---------A
XM_011174635.1      ------AATATTCACAAACCGTATAACCAATTAC----------------------AAGTT
XM_011271310.2      ATGAATAATATTCACGTACCATATAACCAATTGC----------------------AAGTT
XM_012199554.1      ------AATATTCACATACCGTCTAACCAATTAC----------------------AAGTT
XM_011069215.1      ------AATATTCACATACCGTCTAACCAATTAC----------------------AAGTT
XM_015738858.1      ACAAATTCCCATTCGAAATGTCGGAGGGTGATCGAGGCTGCTGACGATTTGCCCGGAATT
XM_015579479.1      ATGAGTCAGCCGCAAACCAGGCA---GAATTCGCTGAAAAAAGG---------------C
XM_012281029.1      ACGGACACAATCGCGGAAACGC---TGCGCGTTCCGTTAAAGAA---------------A
XM_012386439.1      AACAAAGATTCCAAAGAATCGTG---------CGCTCAAAGGAA---------------A
NM_001353392.1      AAGGAAGATTCCAAAAGATCGTG---------CGCTCAAAGAAA---------------A
NM_001190421.1      CCTAATCATTACGATTACGCGTCGTCTGCGAACACTTGGAGGAG---------------A
XM_006618195.1      CCTAATCATTACGAAACGTCGTC---------CACCTCGAGAAG---------------A


XM_008206224.2      ACAGAACCAAA---CGAAGAGGATTTGTATTCTCAAACTATAAAAAAACAGTTGAGAAAG
XM_011174635.1      TCAGACTCTAATAAGATATATGATAAATATTCCAGCGTGATAGTAAATAAAATCAACGAT
XM_011271310.2      TCAGACTCTAACAAGATATATGATAAATATTCCGACGAGATAGTAAAAAAGATCAACGAA
XM_012199554.1      TCAGACTCTAACAGAATATATGATAAATATTCCAAGGAGATGGTAAAAAAAATCAACGAA
XM_011069215.1      TCAGACTCTAACAGAATATATGATAAATATTCCAAGGAGATGGTAAAAAAAATCAACGAA
XM_015738858.1      ACGGGACTGAACGACAATCAGCTAAAATATACGGAGCGAATTCTCAAGAAACTGCAAGAG
XM_015579479.1      GGGGAATCGGTAAAGATATACGACAAATACTCGACGCACATCGTCGAAAAGCTGAACGAG
XM_012281029.1      GAGGATTCCGTGAAAATATACGATAAATATTCGACGCATATCGTCGGCAAGCTGAACGAG
XM_012386439.1      GAAGATTCCGTCAAAATATACGATAAATATTCAGCTTGCATAGCCAAGAAATTGAACGAG
NM_001353392.1      GAAGATTCCGTCAAAATATACGATAAATATTCAGCTTGCATAGCCAAGAAATTGAACGAG
NM_001190421.1      GAAGACTCGGTCAAGATCTACGACAAGTACTCGGCCCGCATCGCGGAAAAATTGAACGAG
XM_006618195.1      GAAGATTCGGTTAAAATCTATGACAAATATTCGCCCCGTATCGCGGAAAAATTGAACGAA
                       *              *       **  *       **        *  *     *


XM_008206224.2      CAAATTAATAATCAGCCGATTAGCGAAGAAAGAAAGAAAAAGATATTAGAATGTAAAAAT
XM_011174635.1      TTGAAGTTCAAGTTAGATGTAGATGCTGAACGAAAGCGTGATATAAAAGCGAGCAATGAT
XM_011271310.2      TTTAAATCCAAGCCGAATGTAGATGCTGAGCGAAAGAATGACATAAAAACGAGTGATGCT
XM_012199554.1      TTCAAATTCAAGTCGGATATAGGTGCTGAACGAAAGCGTGACATAAAAGCGAGTAATGAT
XM_011069215.1      TTCAAATTCAAGTCGGATATAGATGTTGAACGAAAGCGTGATATAAAAGCGAGTAATGAT
XM_015738858.1      TTTAAGGGAGATCGAAAGGTCGCCGCGTTGCGCGAGAGCGCCATCGAAAGGACAGGTCTA
XM_015579479.1      CTGAAGAACAACTCGAACGTAGACAACAAACGGGCGGACGACATAAGGAATAGCGATGAT
XM_012281029.1      TTAAAGAACAATCCGAACGTGGACGACAAACGAGCTCGGGATATAAAAAGCAGCGACGAT
XM_012386439.1      TTGAAGAACAATACAAATGTAGACGATGACCGGACGAATGACATAAAGAACAGTGATGAC
NM_001353392.1      TTGAAGAACAATGCAAATGTAGACGATGGCCGGACGAATGACATAAAGAACAGTGATGAC
NM_001190421.1      CTGAAGGACAATCCGAACGTGGACGACCAACGGGCCAACGATATAAACAACAGCGATGAC
XM_006618195.1      TTGAAGGATAATCCGAATGTGGACGACGAACGGGCGAATGATATAAACAACAGCGATGAT
                       *      *        *           *          **


XM_008206224.2      TTAAATTTACTGCTTTCTGGAAAATTACCACTAGAATCTTTGTGTATAAGTTGTTTAGAA
XM_011174635.1      CTTCGTCGCGCAATGTCAGGAGAATGCGAGATGGAAGCATTATGTTTAAGATGTCTGAAG
XM_011271310.2      CTGCATCGCGTAATATCAGGAGAATGCACAGTGGAAAAATTATGTTTGAAATGTTTAAGG
XM_012199554.1      TTTCGTCGCATAGTATCAGGAGACTGTGATTTGGAAAATTTATGCTTAAAATGTTTGAAA
XM_011069215.1      TTTCGGCGCATAGTATCAGGAGACTGTGATTTGGAAAATTTATGCTTAAAATGTTTGAAA
XM_015738858.1      GCCAGTGAAGAAGTGGACGCTGCGGAACTACTTAAACGGTCCTGTCTCGTGTGCTTAGAA
XM_015579479.1      TTGCGTTCGGTGACAACCGGGAAAACTTCGCTGGAATCGTTGTGCCTGAAGTGTCTGAAG
XM_012281029.1      TTACGCTCCGCGATCACCGGCCGAATACCCTTCGAATCTCTCTGTCTGAAGTGCCTGAAG
XM_012386439.1      TTACGTTCTGCGATGAACGGAAATATCGCGTTCGAATCGTTGTGCCTAAAGTGTCTACGA
NM_001353392.1      TTACGTTCTGCGATGAACGGAAACATCGCGTTCGAATCGTTATGCCTAAAGTGTCTACGA
NM_001190421.1      CTGCGATCGGCGATAAAAGGGGAAATCTCGTTCGACTCGTTGTGCCTCAAGTGCCTTCGA
XM_006618195.1      TTGCGATCGGCTATAAGCGGAGAAATCTCGTTCGATTCGTTGTGCCTAAAGTGTCTACGT
                                    *           *  *       **  *    **  *


XM_008206224.2      TCT------GGTGAAGAGTTGGAGGATCATCCATTTTTTCATGCATCAATGTGTGAAAAA
XM_011174635.1      ATTTATAA---AGGCAAAAAGGAGGAGCATCCGTTCTTTACGGGATCTTTGTGTAAAAAA
XM_011271310.2      TTTTCTAA---AGATAAAATGGAGGAACATCCGTTCTTTATAGGATCTTTATGTAAAGAA
XM_012199554.1      GTTCCTAA---AGGCAAAAAGGAGGAACATCCGTTCTTTGTAGGATCCTTATGTAAAGAA
XM_011069215.1      GTTCCTAA---AGGTAGAAAGGAGGTTCATCCGTTCTTTGTAGGATCCTTATGTAAAAAA
XM_015738858.1      ATCCTTCGAGATGGCAATTGCCAGGAACATCCCTTTTTCGAAGGATCTGTATGCGACGAA
XM_015579479.1      AATTCTGA---CGGACCGATGGAGGCTCATCCGTTCTTCGAGGGTTCTCTGTGCGTCGAG
XM_012281029.1      ATTCCCAAGGACGGGGCTACGGAACTTCATCCGTTCTTCGAGGGATCTTTGTGCAAAGAG
XM_012386439.1      GTTGCTGT---GGGTAAGACGGAAATTCATCCATTCTTCGAGGGATCTTTGTGCAAAGAT
NM_001353392.1      GTTGCTGA---GGGTAAGACGGAAATTCATCCATTCTTCGAGGGATCTTTGTGCAAAAGT
NM_001190421.1      GTTTCCAA---CGACGAAATGGACATTCATCCGTTCTTCGAGGGATCTTTGTGCAAAGAT
XM_006618195.1      GTTTCCAA---CGATAAAATGGACATTCATCCGTTCTTCGAGGGATCTTTGTGCAAAGAT
```

*Figure C. 6 Continued. Multiple aligned sequences to find portions of conserved DNA. The highlighted area represents the sequence selected to create the degenerate primers for Dnmt3. The stars under the sequence signal the conserved bases in all aligned sequences.*

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