Early Development and Genetic Engineering in the Lepidopteran model organism *Galleria mellonella*

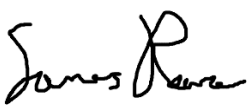
Submitted by James Pearce to the University of Exeter

as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

In September 2022

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.



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Acknowledgements

I would like to thank my supervisors, Professor James Wakefield, Professor Richard Titball, and Professor Joann Prior. Their guidance and support during my doctoral research were essential to both the success of the project and my personal development.

Equally important was the encouragement I received from members of the Wakefield lab, in particular from Dr Karolina Jaworek throughout the four years we have worked together, but more also from Dr Jennie Campbell and Ivan Cañada-Luna. Together with Professor James Wakefield, they have created a fantastic environment to work in at Exeter.

I am extremely grateful for the generous hospitality of the Mosimann and Bopp labs, who hosted me for two weeks in 2019 at Universität Zürich and provided extensive training with CRISPR techniques in zebrafish and insect models.

Lastly, I would like to thank Lina for her incredible patience over the entire course of my studies. I have been truly lucky to be able to count on your support throughout this time.

Abstract

There is a rapidly growing list of publications validating *Galleria mellonella*'s use as an *in-vivo* animal partial replacement model in the fields of infection, immunology, and inflammation.

This is because *Galleria mellonella* larvae exhibit an easily identifiable, but qualitative, biological read-out of such challenges – they produce melanin pigment, turning the larvae from cream-coloured to black. They possess broad susceptibility to microbial pathogens, with pharmacodynamics of drug clearance showing remarkably similar patterns of drug clearance to humans.

Moreover, individual larvae can be precisely dosed by injection, their maintenance is straightforward and, in contrast with competing non-mammalian systems, such as zebrafish, *C. elegans* and *Drosophila*, they can be reared at 37°C, facilitating research into both normal cellular kinetics of biological processes and host-pathogen interactions.

Unlike these other model organisms however, *Galleria* is not currently genetically tractable and lacks detailed protocols for molecular tools or in depth knowledge about its biology. This thesis project describes work done to develop an embryonic microinjection pipeline and better the understanding of preblastodermal development for this organism. In addition, robust protocols for the insertion of new genetic material via PiggyBac mediated transposition, and gene knock-out via CRISPR/Cas9 mediated mutagenesis are described for the first time in *Galleria*.

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Definitions

AA	Amino acid
AMP	Antimicrobial peptide
ASPA	Animals (scientific procedures) act 1986
*Att*B	Bacteria attachment site
*Att*P	Phage attachment site
BF	Brightfield
Cas	CRISPR associated protein
Cascade	CRISPR associated complex for anti-viral defence
CFU	Colony forming units
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
ds	Double stranded
DSB	Double stranded break
EST	Expressed Sequence Tag
FACS	Flow assisted cell sorting
GFP	Green fluorescent protein
GMO	Genetically modified organism
HDR	Homology dependent repair

His	Histone
HSE	Heat shock element
IHC	Immunohistochemistry
IMPI	Insect metalloproteinase inhibitor
iPCR	Inverse PCR
ITR	Inverted tandem repeats
LD50	Lethal dose required for 50% mortality
MAP	Microtubule associated protein
miRNA	micro RNA
NHEJ	Non homologous end joining
nt	Nucleotide
PAM	Protospacer adjacent motif
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
piRNA	PIWI interacting RNA
PO	Post oviposition
PPO	Prophenoloxidase
PRR	Pattern recognition receptor
RISC	RNA-induced silencing complex

RNAi	RNA interference
RNP	Ribonucleoprotein
RT	Room temperature
sgRNA	Single guide RNA
ssODN	Single stranded oligodeoxynucleotide
TNF-α	Tumor necrosis factor alpha
TALEN	Transcription activator like effector nuclease
tracrRNA	Trans acting CRISPR RNA
Tub	Tubulin
UAS	Upstream activating sequence
WT	Wild type
ZFN	Zinc finger nuclease

Chapter 1

Introduction

Until *in vitro* technologies advance to a level where we can artificially replicate and mimic human systems in culture, the use of animals for medical scientific research is sadly a necessity. Research using organisms is vital if we are to be able to accurately understand and solve the complex biological problems that pose a threat to the persistence of our own species. Over the decades this has resulted in a wide range of model organisms that allow us to simplify and replicate various aspects of human biology.

A relatively new addition to these model systems is the greater waxworm moth, *Galleria mellonella* (henceforth referred to as *Galleria*). This moth's immunity and response to infection has been studied since the 1950s, however in the last decade focus has shifted to it as a model for studying human relevant pathogens. *Galleria* is advantageous in this role due to unique aspects of its lifecycle and physiology, yet a lack of genetic and molecular tools and detailed knowledge of its biology are hampering it's wider uptake.

In this doctoral thesis I describe my work to develop tools to improve the versatility and applications of this model and characterize the developmental and cellular biology during early embryonic development.

1.1 *Galleria* Life History

1.1.1 Distribution

Galleria mellonella, also known as the greater waxworm moth or honeycomb moth, is a small grey-brown moth and member of the snout moth family (*Pyralidae*). The larvae of this moth are commercially important insects both as a pest of wild and domesticated bees, and as a for use as fishing baits and live pet foods. Distributed worldwide, *Galleria* are found on every continent bar Antarctica and are adaptable to a wide range of climates. A study by Hosni et al 2022[1] found they were able to survive in conditions where annual mean temperatures and rainfall ranged from 5-28C and 0-2500mm respectively.

1.1.2 Life Cycle and Morphology

Galleria, like all lepidopterans, are holometabolous and begin their life as an egg. These are laid in clusters by gravid females, where they are deposited in crevices or imperfections in a surface on or near a beehive. The eggs are a very pale pink when first laid before turning to a cream white as the egg develops and finally a darker off white as the embryo develops into the pharate larvae[2]. Eggs are spherical or ovoid shape, and roughly 0.5mm in diameter on their longest axis [2–4]. The embryo is covered by a hard chorionic layer with a wavey scale like appearance dotted with aeropyles and with the micropyle present on the anterior pole [3]. When first laid, the chorion is very soft, however hardens within the first hour. The ovipositing female covers the embryos in a mucus like secretion that dries to glue the individual egg both to the oviposition substrate and to other eggs in the same clutch.

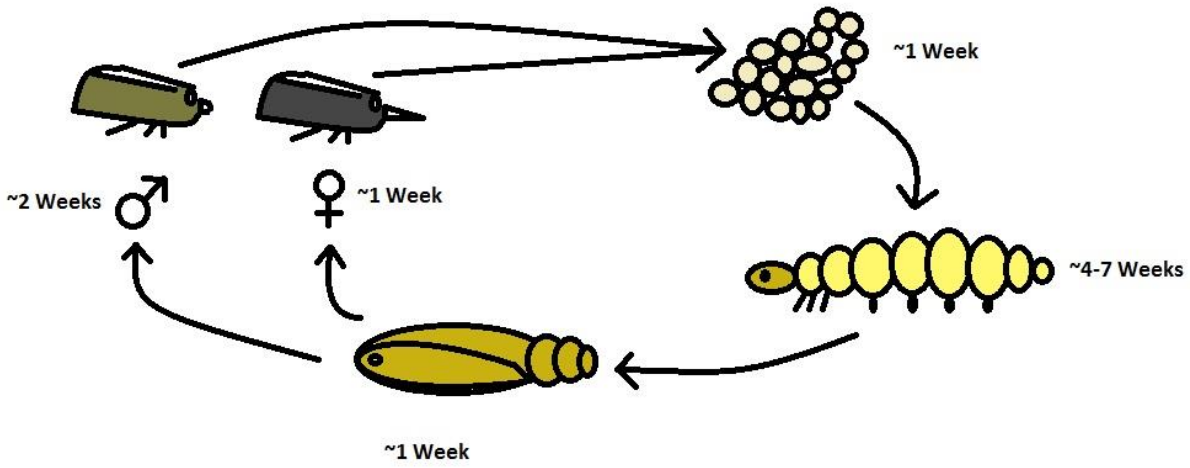

Figure 1.1

Approximate developmental timings during the *Galleria* lifecycle under laboratory conditions at 30C.

The time for embryonic development depends on abiotic factors such as temperature and humidity but was reported as 5-8 days at 24-28C by Jorjao[5] and to take around 6 days at 30C[3]. The exact cause of variability in hatching between egg clutches subject to identical rearing conditions remains unclear, but other factors such as sex may play a part. Abidalla[3] reports that the larvae begin to sclerotise and become pigmented between day 5 and 6 of development coinciding with the change in egg pigmentation.

When ready to hatch, the pharate 1st instar larvae chew through the chorion and crawl out, where they immediately begin to feed and spin silk webbing [6]. In the wild they have been observed to initially feed on wax scale[7] or globules of honey and pollen[8] before burrowing into the comb. The 1st instar larvae are about 1-3mm long with visible thoracic legs[4,6] and are a pale cream with a yellow head capsule. As they tunnel into the comb they feed on wax, pollen, honey and discarded moult casings from bee larvae, undergoing 6-9 larval moults[4,9] and the four prolegs becoming visible after the first moult. The reason for the variability in the number of reported moults within the literature is unclear and could be strain specific or due to environmental factors, since exposure to cold shock or toxins has been shown to induce supernumerary moults [10,11]. The larvae grow most in mass in their final two instars reaching from 18-24mm in length, with their colour changing from a pale cream to an off white to a light grey and their head capsules becoming significantly darker. Larval development time depends on biotic factors such as larval density and abiotic factors such as temperature and nutritional value of the comb, but normally takes between 6-7 weeks[6,9]. So far it has not been possible to determine the sex of an individual at larval stage.

Upon reaching their final instar, larvae tunnel to the edge of the comb or hive where they begin to spin a cocoon. They have been observed to aggregate and investigations by Kwadha et al[2] have shown that decanal released from larvae and cocoon headspaces seems to be the main semiochemical responsible. In the cocoon spinning process a flap is left open at the anterior end for the adult to emerge from and during this process the larvae become sessile and begin to pupate. Pupae begin a very light yellow, changing to a very dark brown over a period of about a week with females being slightly longer and larger than males[2,12]. It is now possible to sex individuals at this stage, with females showing a notch in the sclerite of their 8th segment which is absent in males[12].

Adult moths eclose fairly synchronously during two periods of the day, 6-11am or 5-10pm, normally within an hour of each other and run out of the hive before expanding their wings and seeking shelter once dark[13]. Adults are dimorphic with females usually slightly larger and darker in colour, being a darker brown abdomen and thorax and darker grey wing scales, compared to the lighter colourings seen on males. In addition, females have protracted labial palps giving the appearance of a snout (hence snout moths), whereas the male's palps curve back over themselves giving a more blunted appearance[12] (Fig 1.2). *Galleria* have an unusual mating system, whereby the male attracts the female by sound produced by tymbals located on their tegulae[14] (Fig 1.3). This causes the females to fan their wings, in turn causing the males to release a sex pheromone initiating the onset of mating. Svensson et al[15] found that, of compounds collected from adult headspaces, nonenal, decanal, undecanal and a novel sex semiochemical 5,11-Dimethylpentacosane elicited responses from *Galleria* antenna. A mixture of nonenal, undecanal and 5,11-Dimethylpentacosane was required to elicit significant

taxis. After mating had occurred, gravid females flew to bees' nests to oviposit on substrates proximal to hives, and were most active during the early phases of the onset of darkness [2,13]. Adults do not feed, instead surviving off energy reserves built as larvae, with males live approximately twice as long as females in the wild (21 vs 12 days), although the exact length depends on abiotic factors.

More thorough reviews of the biology of *Galleria* are available than summarised here for early and middle-late embryogenesis[3,16], and larval and adult morphology and behaviour (Smith 1965, Kwadha et al 2017, Nielsen et al 1977, 1979, Ellis et al 2015).

Figure 1.2 *Galleria* adults displaying sex dimorphism

Male and female *Galleria* adults display various dimorphic features with females usually larger and darker. Most notable however is the more pointed snout-like structure formed from the females' labial palps (A) as opposed to the more blunted snout found in males (B).

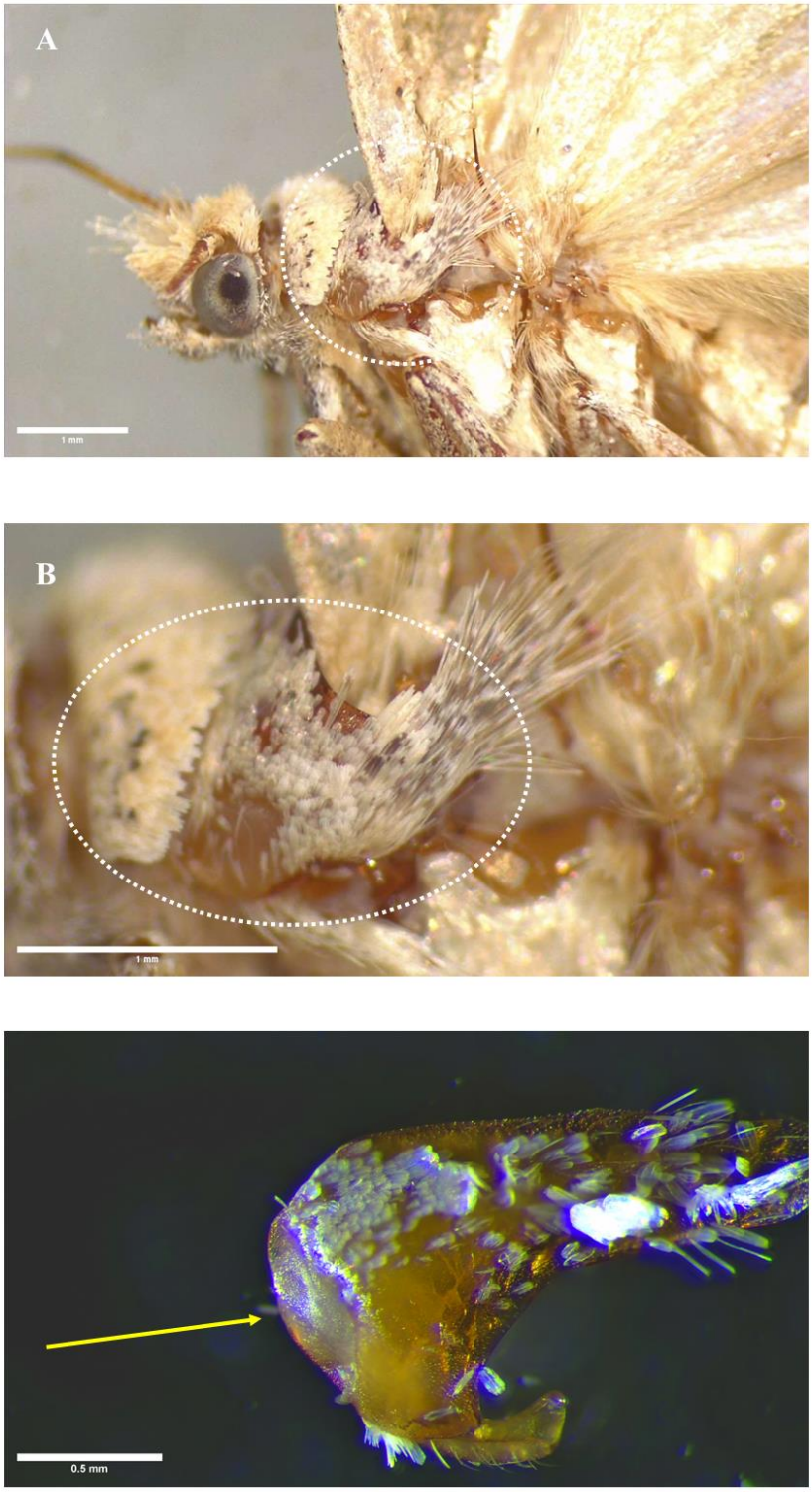

Figure 1.3 *Galleria* Tymbals are located at the base of the tegulae

The tegula is a small sclerite above the base of the wing. It is shown *in-situ* at the base of the forewing on the same adult male in images A & B (dotted circle). In image C, the tegula has been dissected and scales have been removed to reveal one of the tymbals at the base of the sclerite (yellow arrow).

1.2 *Galleria* as an infection model

1.2.1 The *Galleria* immune system

Asides from commercial use as bait and live food for pets, *Galleria* is most commonly used as a model for microbial infection, particularly for human relevant pathogens. Whilst perhaps initially counterintuitive, the physiology of *Galleria* and similarities between mammalian and insect immune systems make *Galleria* a suitable replacement for infection studies traditionally carried out using rodents. *Galleria*, like all insects, do not possess an antibody mediated adaptive immune system but instead rely on a combination of immune pathways (reviewed Jiang et al 2010, Wojda 2017, Pereira et al 2018, Sheehan et al 2018, Wright and Kavanagh 2022[17–21]) that closely resemble mammalian innate immunity.

The binding of pathogen associated molecular patterns (PAMPs) on the surface of microbial pathogens by pattern recognition receptors (PRRs). Different PRRs, such as peptidoglycan receptors, β1-3 glucan receptors and lipopolysaccharide receptors can recognise a wide variety of microbes [22,23], including gram positive and negative bacteria and fungi, in turn activating downstream pathways. These Toll and IMD like pathways, similar to those found in *Drosophila melanogaster (henceforth referred to as Drosophila)*, are equivalent to IL-1 and tumour necrosis factor α (TNF-α) pathways seen in mammals[24] and activate *Galleria*'s cellular and humoral response. Whilst Toll receptors have been shown to be involved in dorso-ventral patterning in both *Drosophila* and *Tribolium* embryos[25], their role in Lepidopteran embryogenesis and pattern formation has not been described to date. However the presence of certain Toll transcripts only in ovarian and embryonic EST databases in *Bombyx* suggests they may have a roll [26].

The cellular response consists of a variety of different cells called haemocytes that circulate within the body cavity suspended in the haemolymph. These cells functionally resemble various types of white blood cells found in mammals and are involved in encapsulation, phagocytosis, nodulation, and aspects of the melanisation cascade. There are currently thought to be at least 5 different types of haemocytes: Precursor cells or prohemocytes, granulocytes, plasmacytes, oenotocytes and spherulocytes[27] which have been characterised at a subcellular level by Salem et al[28] and Wu et al[29]. Plasmacytes and granulocytes are the most common type found within the haemolymph and are the primary drivers of encapsulation, nodulation and phagocytosis[30]. Oetonocytes are thought to be involved in the melanisation response since they contain components of the melanisation cascade [31], however the function of spheruloctes is so far unknown.

The humoral response consists of multiple components – such as antimicrobial peptides (AMPs), reactive oxygen and nitrogen species, and both melanisation and opsonisation via the prophenyloxidase (PPO) cascade. These defensive molecules are primarily produced by the fat body (a highly metabolic organ equivalent to the insect liver[32]) and the haemocytes, whereupon they can be secreted into the haemolymph or the site of infection. AMPs tend to be small cationic peptides, such as defensins and lysosymes, that are able to damage membranes in microbial pathogens and thus help clear infections[33]. Important AMPs include cecropins, moricins and gloverins, gallerimycin and galiomycin as well as the insect metalloproteinase inhibitor (IMPI)[18] – with some having activity to only specific classes of pathogen and activated by different immune cascades. These cascades also cause the release of reactive oxygen and nitrogen species, although the mechanisms by which this happens are currently unclear, which help fight infection

by inducing oxidative stress to pathogen. They are primarily released by haemocytes [34]and other cell types such as intestinal epithelial cells[35] but can also be found free floating in the haemolymph.

A key component of the innate immune system in mammals is the complement cascade, a highly regulated cascade which promotes inflammation and activates protein complexes that are able to directly attack and opsonise pathogens. This closely resembles the melanisation in *Galleria*, which is a rapid response to wounding or infection mediated by the PPO cascade. This is initiated by the conversion of phenolic compounds to quinines, via an enzyme known as phenoloxidase, which are then later polymerised to melanin. PPO is present mainly in oetonocytes as the inactive precursor PPO, and activation of the serine protease cascade by PGRs leads to activation of this enzyme[20]. Oetonocytes are stimulated to release components of this melaninization pathway into the body cavity which allows the melanisation and encapsulation of pathogens within the body cavity or at the site of wounding[19]. This enzymatic process releases reactive species which are themselves cytotoxic and works in tandem with coagulative proteins and those with opsonic functions such as apolipophorin III and haemolin to recruit phagocytic cells to sites of infection and engulf potential pathogens [21].

The similarities between mammalian and *Galleria* immunity have meant that *Galleria* has been used to study host-pathogen interactions since the 1950s[36–40]. Additionally its larval life history, by which it develops in beehives, means it is well adapted to their internal temperature of 32-36C[41] which approximates that of human body temperature. This provides an advantage to studying both mammalian pathogens which require incubation at 37C and those with temperature dependent effects. To date it has been validated via virulence studies on a wide range of microbial

adversaries including fungal and both gram positive and negative bacteria, as summarised by Pereira et al[42]. *Galleria* can also be used for toxicity testing and studying the action of antimicrobials[43,44] with LD50s broadly corresponding to those seen in mammals[45–48] which greatly opens up their use as a partial replacement for rodents in pharmacological discovery. The fact they are not currently covered by the Animals (Scientific Procedures) Act of 1986 offers a more ethical choice for researchers looking to conduct microbial research in animals, which combined with the low cost and ease of availability, maintenance and use within the laboratory makes them a very promising model organism.

1.2.2 How *Galleria* is used as a model

Galleria, thus far, has mainly been utilised in the larval stage for infection studies – normally cited as between 4th and 6th instar and of a weight between 180-300mg[49] although it is not completely clear if these two measures correlate. Larvae that have been purchased from commercial suppliers are normally stored in total darkness at a temperature of 15-20C to slow down development but are brought back to room temperature before experiments begin. Larvae should not be stored at lower temperatures as excessive cold shock can prime the immune system before infection assays begin[50]. To try and reduce variability in assays, melanised or sickly larvae should be removed from the pool of available insects. Additionally, some commercially available larvae are surface sterilised (Tru-Larv) to help reduce experimental variability from external microbial flora, but this may be replicated by the researcher by briefly washing larvae in ethanol solutions ranging from 50-100%.

Healthy larvae may then be infected with the researcher's pathogen of choice, most commonly via injection through the proleg[51] using a Hamilton syringe but force feeding protocols are available[48,52,53]. *Galleria* are commonly temporary restrained physically whilst injecting, for example by bending them backwards over a pipette tip[51], however alternative restraint methods[54] or even CO_2 anaesthesia will work. Due to their large size and open circulation system *Galleria* it is possible to inject quite large volumes into the cavity, and doing so above a piece of filter paper allows the operator to ensure no leakage out of the proleg has occurred. They may then be transferred to a petri dish and incubated at the desired temperature.

To investigate the effects of a pathogen on larvae, survival assays can be used. Simply put, this typically involves injecting multiple replicates of groups of ~10 larvae with a known concentration of microbial agent and monitoring their health for roughly a week to 10 days. The time taken to kill the larvae depends on the pathogen itself and both the concentration and volume injected, requiring researchers to optimise this figure (between 10^4 and 10^6 CFU is a common range). PBS injected negative controls are commonly used although the exact nature of controls used will depend on experimental design. During the assay, larvae commonly will melanise turning dark both locally at the injection site and varying from light grey to black across the entire body as the insect's melanisation cascade is activated in response to pathogens. Larvae will often become sessile as the infection triggers serine proteases[55] which in turn causes the activation of cytokine like molecules called ENF peptides[56,57]. As well as upregulating inflammatory pathways and other immune responses the ENF factors result in reduced larval growth, cause larvae to become sessile and delay pupation[58].

As the larvae become sessile, it can sometimes therefore be difficult to tell when a larvae is actually dead which can in turn influence calculated LD50s and make replication between experiments, individual researchers and groups more tricky. Efforts were therefore made by Loh et al[59] to create a standardised scoring system for health during a larval infection assay. This takes into account 4 mains factors: motility, cocoon formation, melanisation and mortality. It seeks to grade these in a repeatable fashion to give a score out of 10 which could used to quantify larval health. Whilst this is a useful improvement upon live/dead scoring, issues still remain as it does not allow comparison between pathogens, which may differentially activate the PPO cascade. This means that larvae can melanise within minutes or over several days depending on the pathogen.

Throughout the survival assay larvae can be co-injected with a therapeutic to investigate its actions on pathogens. Additional data may also be gathered during or at the end of the assay by extraction of haemolymph through careful extraction through the proleg using a fine needle. The haemolymph and cells within it can then be analysed via IHC, FACS or flow cytometry to investigate pathogen clearance or persistence. To better study pathogen localisation within particular tissues, whole larvae can be dissected, or paraffin embedded to allowing a better visualisation of particular tissues or transverse slices of whole mounts.

1.2.3 Limitations of the *Galleria* model

Despite the overall benefits and increasing uptake and use of this model by the wider scientific community there are however some issues that are researchers currently face.

Foremost of these is a lack of standardised protocols. Multiple authors have released manuscripts to try and standardise methods using *Galleria* for topics ranging from rearing[5,6,60] to storage and handling[50,61–63] or infection assays [64,65] as small changes in these protocols can have large effects on assay outcomes. Yet no centralised repository for techniques or methodology exists for *Galleria*, as there is for almost every other commonly used model.

Another is that the majority of lab *Galleria* are obtained from commercial suppliers. This is a huge benefit of the model for most microbial researchers as they do not need to invest the time, money, and space to maintain a colony and larvae instead are available on demand and cheaply in the post. What researchers gain in convenience however, they lose in their ability to control the quality of the larvae. Differences in biotic and abiotic conditions during larval development and shipping can have an impact on infection outcomes[5,66] and it is not immediately apparent if a larval supplier supplements their diet with antibiotics. Careful larval screening and handling or storage procedures are therefore needed before an assay to ensure maximum replicability between different groups or suppliers.

Equally, whilst our understanding of developmental biology of *Galleria* is increasing and is aided by previous studies on the lepidopteran model *Bombyx mori,* there are still lots of shortcomings to our knowledge of even basic morphology. As an example, neither embryonic nor larval stages can be sexed on external morphology alone, leading to an inability to investigate sex linked effects. The use of a range of larval sizes used also poses a problem as a larva's proximity to moulting or pupation during development can result in widespread hormonal and proteomic differences between experiments. This in turn requires greater sample sizes to be used to account for this variation.

Happily, there is now an annotated genome available for *Galleria* submitted by the University of Urbana Champaign, Illinois (GCF_003640425.2) after it was first sequenced by Lange et al in 2018 (GCA_002589825.1) [67] and again by Kong et al in 2019 (GCA_004355975.1) [68], with the latter two unannotated. The assemblies for these however vary in size by ~180Mbp (from 398-578Mbp) , with the smallest 70% of the size of the largest, indicating that there may be a huge amount of genetic data missing from the reference genome. Genetic assembly mapping is currently limited to scaffolds for the reference genome and contigs for the other two rather than chromosomes, although this will likely change as the annotation of assemblies improves. Whilst more detailed information for *Galleria* is lacking, fortunately annotated genomes at chromosomal level are available for *Bombyx mori* (https://kaikobase.dna.affrc.go.jp/) and several other Lepidopterans (http://lepbase.org/). There are now also a wide variety of transcriptomes available (https://www.ncbi.nlm.nih.gov/biosample/?term=(txid7137)) for different life stages (embryo, larvae, pupal, adult), tissues (gut, fat-body), and infection statuses in *Galleria* which will hopefully help us to better understand the fluxes in gene expression during development and immune challenge.

One of the main areas that *Galleria* lags other commonly used models though is its genetic tractability. *Drosophila* has been used for seminal research in animal genetics and the ease at which this and other commonly used models such as zebrafish (*Danio rerio*) can be genetically altered vastly increases both the applications and information that results from animal experimentation. Indeed, genetic tractability is valued in rodents too. According to the UK statistics for scientific procedures on animals, 41% of the experimental procedures performed on mice and rats for immune, infectious disease and toxicological testing across basic,

applied, and regulatory use in 2020 used genetically engineered animals. The genetic toolkit in *Galleria* is so far limited to RNA interference (RNAi)[69], which showed that direct injection of double stranded RNA directly into the body cavity was enough to significantly silence expression levels of IMPI in several tissues. At the time of writing however, no publications have demonstrated the feasibility of utilising existing molecular techniques to generate transgenic or otherwise genetically modified *Galleria*, despite the advantages it could confer to this model.

1.3 Techniques for generating genetically modified organisms (GMOs) and modifying gene expression

The ability to genetically modify organisms is extremely useful to scientific study. It describes how we can modify an organisms genome by incorporating new exogenous genetic material into it, a process known as transgenesis, or by modifying existing genes within that genome. These changes elucidate far more information than would normally be available by biochemical or molecular techniques and allows the monitoring of dynamic processes within an organism and interrogation of genomic function to unravel the complex interactions that make up living systems.

The first GMO to be created was a mouse in 1974 by Jaenisch and Mintz[70], with the first stable germ line transmission in mice in 1981[71]. Shortly after the first transgenic *Drosophila* was developed[72], with nematodes[73], *Xenopus*[74] and zebrafish[75] following in quick succession. A wide variety of genetically modified insects have now also been developed across several orders besides Diptera including Coleoptera[76], Lepidoptera[77] and Hymenoptera[78]. More recently approaches involving genetically engineered insects have been employed to try and solve issues such arthropod borne disease and crop damage[79–81].

Exact methodologies for generating GMOs vary depending on the species and its life history. For the most part it boils down to introducing nucleic acid mutagenic agents intracellularly when the organism is at a single cell stage, commonly at embryonic stage just before or after the two pronuclei join to form a zygote during fertilisation. These nucleic acids, or the proteins they encode, are then able to introduce breaks in genomic DNA and cause deletions, small alterations or even add large insertions in the organism's genetic code. Physically this is a lot easier to manage in oviparous

versus viviparous organisms such as mammals but is possible provided techniques for safe embryonic extraction from within the female exist. The most common way to introduce these mutagens is then to use a small, specialised needle to bypass the embryonic barriers and membranes and inject them directly into the cytoplasm, however alternative methods including electroporation or viral infection may be viable depending on the organism[82]. Once introduced the mutagenic agents are then able to alter the chromosomal DNA in the organism. Provided that this happens early in development, there is a strong chance that these mutations will be present in the germ line and be passed on to the next generation. If mutations were to occur later in development however, after the zygote has divided and the germline tissue presumed to have formed, the likelihood of these mutations being present reduces with each successive round of cell division.

Since there are multiple different genetic methodologies available today (Fig 1.4) this next subchapter will focus on the various techniques commonly used in insects.

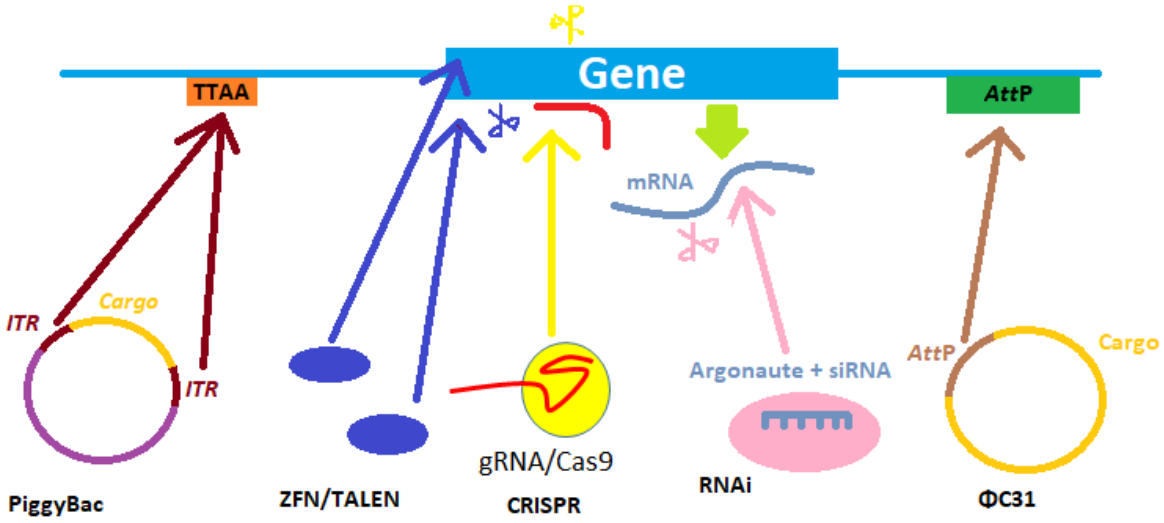

Figure 1.4 Overview of transgenic methodologies

Multiple technologies exist for genetic engineering. Some like ΦC31 integrase and PiggyBac can used to integrate large cargos within the genome at either a targeted or semi-random locus respectively. Others such as ZFN/TALENs or CRISPR are adept at inducing double stranded DNA breaks at targeted loci. RNAi, whilst not technically genetic engineering, is able to degrade messenger RNA transcripts and therefore temporarily silence gene expression.

1.3.1 RNAi

RNAi was discovered in 1988 by Fire et al.[83] and represented a breakthrough in the study of non-coding RNAs. This RNA mediated gene silencing is present in all eukaryotes and leads to degradation of RNA products within the cytoplasm. This is affected by argonaute proteins bound to very short single stranded RNAs (siRNAs), and in insects is the primary antiviral defence. In *Drosophila*, RNAi mediated degradation can occur via two pathways. Both involve binding of double stranded sections of RNA by RNAse III enzymes, know as Dicer 1 and 2, and their subsequent trimming to double stranded RNAs or ~20nt[84,85]. One of the RNA strands is then incorporated into an RNA-induced silencing complex (RISC) before binding and degradation of complementary RNA by the RISC.

Endogenously produced microRNAs (miRNAs) are processed in the nucleus to form short stem-loop structures with 3' overhangs before export into the cytoplasm. The loop structure is cleaved by a Dicer1-Loqs complex creating an overhang on the second strand and finally a single strand is incorporated into an argonaute 1 containing RISC. Exogenous dsRNA molecules present in the cytoplasm, either as a result of viral infection or from scavenger mediated endocytosis, are degraded into short 20nt dsRNA molecules known as small interfering RNAs via the Dicer 2, leaving 3' overhangs on both strands. The R2D2 protein associated with Dicer 2 to separate the strands before loading onto the argonaute 1 RISC and further modification of the siRNA guide. Both RISC complexes then degrade complementary single stranded RNAs within the cytoplasm, thus reducing the expression of their transcripts (Reviewed in more detail by Schuster et al 2019, Vogel et al 2019[86,87]).

RNAi is a very powerful tool in insect, as by injecting or expressing short double stranded or hairpin RNA molecules, this gene silencing pathway can be hijacked to selective degrade endogenous transcripts. This has been used to great effect in *Drosophila* via genome wide loss of function RNAi screens, provided large amounts of experimental evidence to support gene functions. However not all insects are as susceptible as others – Lepidoptera in particular appear less affected. In *Ostrinia furnacalis*, a species closely related to *Galleria*, the Lepidopteran specific *up56* gene was found to be swiftly upregulated in response to double stranded RNA. The gene product is able to digest ssRNA, dsRNA and dsDNA and the rate at which transcripts appear in the cytoplasm is much quicker than those of *Dicer*. When this itself was knocked down *in vivo*, a seven-fold increase in RNAi efficiency was seen[88]. A systematic review by Terenius et al.[89] found that RNAi efficiency in Lepidoptera varied greatly depending on species, method of dsRNA introduction, tissue, and type of targeted genes. Targets involving tissues and effectors constituting the immune response seemed to be responsive to RNA, and it is this which has been successfully knocked down in *Galleria*[69].

1.3.2 Transposon based methodologies

Perhaps the most common of transgenesis techniques are transposon-based methodologies. These are based on transposable elements, DNA sequences that can alter their location within a genome. Transposon based technologies use class II transposable elements, ones in which the DNA mobile element is translocated via a specialised enzyme that excises the mobile element via special recognition sequences on either end. The enzyme that performs this task can be encoded within

the transposon or elsewhere in the genome. Transposon based techniques manipulate this by placing genetic cargos between the two recognition sequences and using a separately transcribed transposase to move them randomly into the genome. Transposable elements are present in most living organisms, with a wide variety described[90–92], but the ones most commonly used in insects are P-element transposition and PiggyBac transposition.

The P-element transposon was initially isolated from the *Drosophila*, as the cause behind hybrid dysgenesis. This is a phenomenon in which wild type (WT) males crossed to laboratory females results in sterile offspring due to mutations, chromosome breaks and rearrangements, yet the reciprocal cross is healthy. This paternal contributing element (hence P-element) is believed to have been transferred horizontally to *Drosophila melanogaster* sometime in the 20th Century as it is not present in most laboratory strains. Wild type strains strongly repress these mobile elements, and this seems to be epigenetically mediated by piRNAs in the ovary cytoplasm[93,94]. The presence or absence of P-elements is now more commonly referred to as P (P-element present) or M (P-element absent) genotypes, with the sex specified in crosses due to the maternal deposition of the aforementioned factors.

The transposase itself requires a variety of sequences to be able to excise genomic content including a 31bp inverted terminal repeat (ITR), a 10bp transposase binding site and 11bp subterminal repeat[95]. The transposase itself however does not bind to these sites, and instead are bound site specifically by a *Drosophila* encoded protein, Inverted Repeat Binding Protein (IRBP)[96]. Co-injection of two plasmids, one containing the transposase and the other the cargo flanked by the recognition sequences, into the posterior pole of a *Drosophila* embryo allows temporary

expression of the transposase and mobilisation of the cargo into the pole cells. As with all transposons, the locus at which it integrates is random and therefore it is possible for it to disrupt endogenous gene function and have other knock-on effects on viability[97]. Whilst not widely used outside *Drosophila,* P-element like sites and transposases have been identified in other organisms too such as humans and zebrafish[98] and are able to mobilise *Drosophila* elements *in vitro*. The efficiency of Drosophilid P-element transposase *in vivo* in non Dipterans however, appears unclear[99,100]. The lack of Drosophilid IRBP and other endogenous factors and the presence of localised sources of transposase in some organisms could lead to low integration efficiency and unstable integration, which might explain their limited succes outside *Drosophila*. Instead, other forms of transposase are more widely used.

The most commonly used Transposon system is the PiggyBac transposon, first identified as insertions into serially passaged baculovirus in the cabbage looper *Trichoplusia ni*, and characterised in the 1980s[101]. Signature to the transposase was duplication of TTAA sites either side of a pair of inverted terminal repeats. The transposon itself is a 2.5kb autonomous element encoding a single 594 amino acid transposase[102] and similar transposase like sequences have been found in a wide variety of organisms in fungi, plants and animals[103] PiggyBac transposases excise their cargos by cleaving the distal sides of each terminal repeat, producing a single TTAA overhang on each side and allowing hairpins to form on either end of cargo. The donor sites are then seamlessly repaired to leave a single TTAA site. The hairpins are then opened before integration of the donor into the new TTAA site within the host genome[102]. This results in a scarless excision from and integration into the genome. PiggyBac appears to have a preference for open chromatin states

such as gene encoding regions[104], avoiding heterochromatin and mostly travelling short chromosomal distances between transpositions[105]. Similar to P-element transgenesis, PiggyBac mediated transgenesis is conferred by introduction of a genetic cargo located between ITRs and an auxiliary transposase. PiggyBac is valued for its ability to work in a wide variety of organisms and its large cargo size (>200kb[106]), with domestication of its sequence via codon optimisation increasing its suitability to mammalian applications. The insertions themselves seem to be relatively stable between generation[107], with endogenous PiggyBac like transposases failing to mobilise PiggyBac elements in the closely related Lepidopteran *Bombyx mori*. Efficiency of integration appears to vary greatly depending on the organism, constructs used and technique however [108–111] and despite being a more versatile system, PiggyBac still suffers from the same issue as P-element transgenesis in that insertions are random and can disrupt endogenous gene function.

1.3.3 ΦC31 Integrase

P-element transposition in *Drosophila* has more recently been surpassed by a technique utilising ΦC31 integrase. This is a phage derived site-specific recombinase technique, similar to the bacteriophage derived Cre-Lox methodology commonly used in mammalian systems, that allows insertion of exogenous genetic material at a precise landing site within the genome.

The serine integrase itself mediates recombination between small DNA sequences known as the bacterial attachment site (*Att*B) and the phage attachment site (*Att*P). Unlike Cre recombinase, ΦC31 integrase only mediates integration and not excision

which means that recombination is unidirectional. Transposon based methodologies are initially used to integrate an *Att*P landing site into the genome, whose chromosomal location can be inferred by crosses and identified via inverse PCR (iPCR) or sequencing. Co-injection of the integrase and a plasmid containing the *Att*B site leads to recombination between the two sites and integration of the entire plasmid into the genome with *Att*L and *Att*R recombinant sites flanking either side plasmid sequence.

This technique has several advantages over transposon-based methodologies for generating transgenic organisms. Recombination happens at a high frequency [112] and occurs at a known locus within the genome, although it has been shown that recombination is also possible at low frequency at endogenous pseudo *Att*P sites in mammalian systems and *Drosophila* [113,114]. The known landing site can be used to ensure exogenous genomic cargo is inserted where it will not be greatly affected by chromatin state or disrupt neighbouring genes or regulatory elements (and vice versa).

Since *Att*P sites are small sequences of ~40bp they are easy to add on to existing transposon-based cargos or integrating via DNA repair methodologies to targeted loci after induced DNA breaks. Use of the ΦC31 integrase technique has be described in Lepidoptera[115], but the overall lack of Lepidopteran specific ΦC31 donor vectors and widespread ubiquity of PiggyBac donors has likely limited its uptake. The ability to integrate large donor cassettes at known genomic loci could, however, be a distinct advantage for *Galleria* as transgenic techniques are developed and genome annotation improves.

1.3.4 Gal4/UAS systems

Whilst not a method for generating new transgenic organisms it is worth noting a highly versatile expression system used extensively in *Drosophila*. GAL4/UAS is a bipartite expression system that allows separation of promoter activity from downstream reporter expression. First used in mammalian systems in 1988[116] and in *Drosophila* in 1993[117] it uses the yeast transcription activator protein GAL4 to activate its corresponding effector, the upstream activation sequence (UAS).

The promoter activity of a transgenic construct containing GAL4 determines where and when GAL4 protein is made, and thus determines where and when the effector downstream of the UAS sequence is expressed. By developing transgenic driver lines which contain only GAL4 and responder lines containing only the UAS it is possible to maintain lines whose genotype would otherwise have low fitness or sterility due to severe deleterious effects from continual co-expression of the GAL4/UAS system. Crossing these two lines together, however, produces offspring which strongly express the genomic sequences downstream of the UAS in a temporal and spatial pattern determined by the sequence upstream of the GAL4 driver.

Since individual GAL4 and UAS constructs are not linked to the same line, they can also be used in combination with other GAL4 or UAS lines, greatly increasing the versatility of lines. Beyond its use in *Drosophila*, GAL4/UAS has been implemented in other insects (including lepidoptera) [118–121]and zebrafish[122] and would surely have multiple applications in *Galleria*, especially due to ability to negate the negative fitness implications associated with overexpression or knockdown of immune related genes.

1.3.5 ZFN/TALENs

Zinc finger nucleases and transcription activator-like effector nucleases are endonucleases that are able induce DNA breaks at precise genetic loci. These chimeric proteins combine DNA binding domains that give them specificity to particular sequences and domains with nuclease activity that produce double stranded breaks (DSB). The ability to alter the specificity of DNA binding domains in these proteins by altering their amino acid sequence allows targeting of particular DNA sequences within loci. These specific DNA breaks can then be used add or remove gene function inducing knock-in or knock-out mutations. The versatility and specificity of these systems offers a useful tool for investigating how loss or gain of gene function can affect an organism.

Zinc fingers nucleases contain multiple Cys_2-His_2 zinc finger domains, the most common eukaryotic DNA binding domain. Different zinc fingers are able to bind different 3bp regions within the major groove of a DNA double helix[123], and by engineering new zinc finger constructs containing multiple zinc finger domains recognition sequences of 9-18bp region can be generated[124]. These longer recognition sequences allow enough specificity for polydactal proteins to be used to target a single sequence in mammalian genomes[125]. Zinc finger domains are commonly fused to FokI nuclease domains, which function as a dimer and thus two ZFNs are required to generate a single DSB[126]. Genomic DSBs are often repaired by the homology independent non-homologous end joining (NHEJ) repair method. Whilst this method repairs breaks quickly it is error prone and often results in small insertion-deletion mutations (indels) that can disrupt genomic function if they occur within a gene region. Homology dependent repair (HDR) happens at a lower frequency in most organisms, but exogenous HDR templates with homology regions

to the site of the DSB can be used via this pathway to insert novel genetic material at a desired locus. Advantages of ZFNs include high efficiency of cutting and specificity as off target DSBs are rare since they target two slightly different sequences and require both to be bound in order to cut. However, individual zinc finger domains can influence the specificity of others making design trickier and whilst these ZFNs are now available to order commercially, they require 2 new proteins to be synthesised for each target sequence and thus are costly and time consuming to generate at scale.

TALENs are chimeras of a non-specific FokI nuclease fused to TAL effector DNA binding domains. Trans activator-like effectors were originally isolated from proteins secreted by *Xanthomonas* proteobacteria[127] and contain conserved multiple 33-35 AA repeats. Variable residues at positions 12 -13 within each repeat, termed repeat variable diresidues confer binding specificity to a single DNA base[128], and by arranging these AA repeats in different arrays different target sequences can be predictably specified[129]. TALENs induce mutations in a very similar way to ZFNs and have thus have similar advantages and disadvantages. Whilst they remain efficient [130,131]), the additional benefits of this technology are in their simpler design as TALE domain repeats within an array do not affect each other, enabling researchers to reliably predict what sequence a domain will bind to.

More recently, and despite ZFNs and TALENs being used genetically modify a wide range of animas [132–136] the advent of a newer technology, CRISPR, has meant that ZFNs and TALENs have fallen out of favour for targeted mutations. That being said, TALENs still have their place in certain applications and organisms, where their high target cutting efficiency can allows comparable rates of incorporation of large donor templates [137].

1.3.6 CRISPR

The most commonly method for genetic engineering used today, and one which has exploded in applications and scope since its discovery 10 years ago[138], is CRISPR. This system is able to induce DSBs at target sequences using a modular approach system whereby RNA molecules are able to guide a bound nuclease to their complementary DNA sequence.

CRISPR (clustered regularly interspaced short palindromic repeats) has evolved as an RNA guided defence mechanism in bacterial and archaeal defence against viruses[139] functioning as a form of adaptive immunity in these kingdoms. These organisms have clustered short palindromic repeats within their chromosome within which short fragments of foreign nucleic acids are appended, which helps to maintain a cellular memory of past infectious agents. These short fragments, known as protospacers, along with the repeats are transcribed to form long RNAs. Individual protospacer-repeat loci are then processed to short RNAs, known as crRNAs, by CRISPR-associated proteins (Cas) and packaged with other types of Cas proteins. These then circulate around the cell, binding and cleaving foreign nucleic acids with complementary sequences to the crRNAs and thus silencing them[140].

CRISPR systems are broadly divided into two classes, which can then be further subdivided into six different types[141]: Class I involves a large number of Cas proteins which form a complex known as Cascade (CRISPR associated complex for anti-viral defence) to cleave nucleotides, Class II however does not form large CRISPR/Cas complexes, but cleavage is instead mediated by a single effector protein that both binds crRNAs and cleaves the target nucleotide[142]. Of these Class II Cas effectors,

Cas9 was the first to be found but other single effector Cas proteins such as Cas12 and Cas13 have since been discovered.

Cas9 nuclease is unable to cleave complementary targets by binding just a crRNA. Instead, it requires another nucleotide, a trans-activating crRNA (tracrRNA) in order to form the correct ribonucleoprotein (RNP) complex. Target cleavage depends not just on complementarity between the crRNA and the protospacer, but also the presence of a particular nucleotide sequence next to the protospacer – the protospacer adjacent motif or PAM. For Streptococcus pyogenes Cas9 the PAM is the nucleotides NGG, and blunt end double strand breaks are then induced 3-4 bases upstream of this sequence. Jinek et al.[138] found that by altering the protospacer region within a crRNA to be complementary to a particular sequence and assembling this with Cas9 and a tracrRNA to form a RNP complex, it was possible to accurately target and cleave sequences in vitro. They also discovered that fusion of both crRNA and tracrRNA to a single chimera known as a single guide RNA (sgRNA) functioned equally well. Cas12 and Cas13 nucleases are similar but work slightly differently, as not all require tracrRNAs and both the PAM requirements and cleavage patterns differ. Of the commonly used subtypes, Cas12a requires a longer T-rich PAM and cuts in a staggered pattern. Cas13a and Cas13b both target RNA sequences, do not require a PAM and degrade both target sequences and other nearby RNA – known as collateral cutting[143].

The in vitro success of CRISPR mediated cleavage was quickly transferred to in vivo applications[144–147] and has become the de facto choice for gene editing. Similar to ZFNs and TALENs, injection of preassembled RNPs or nucleic acids encoding sgRNA and nuclease into the pronuclease induces DBS in the genome. These are repaired most frequently by the error prone NHEJ pathway which often induces

indels and can disrupt gene function (Fig 1.5), although large deletions of over 100kb may also be induced by using multiple guide RNAs targeting the same locus (song 2017). This technique may also be used to integrate exogenous DNA from donor repair templates via HDR, although efficiency can be an issue due to prevalence of NHEJ [148]. This may be improved by removing or suppressing NHEJ machinery[149], expressing Cas9 protein in the germline[150], or for smaller knock-ins by utilising single stranded oligo deoxynucleotides (ssODNs) repair[151]. There is evidence also that concurrent cleavage of both donor and target by the nuclease can increase efficiency[152,153] and that this can be further improved by using microhomology arms[154].

To date CRISPR has been used in a wide variety of insect species and at least 6 orders [155–162] and this list will continue to expand. CRISPR is also the most promising of the gene editing technologies currently available. It has a large advantage over ZFN and TALEN based techniques due to the adaptability of the system – a single nuclease is needed and guide RNAs can be synthesised or both purchased cheaply from commercial suppliers rather than having to engineer new proteins or constructs each time. Advances in CRISPR tools also means that CRISPR interference (CRISPRi) can function as a replacement for RNAi, albeit via inhibition of mRNA translation by steric hindrance versus the actual degradation of mRNA. Where CRISPR currently lags is knock-in efficiency vs transposase/ ΦC31 integrase and availability of donor constructs, and the reliability of TALEN based techniques for larger targeted knock-ins. However this will surely change in the near future, or combined with such techniques to knock-in landing sites for larger constructs at known genomic loci. The ability to target almost any locus in the genome and place

reporter under endogenous control is extremely appealing versus random integration and the need for lengthy reconstitution of regulatory sequences.

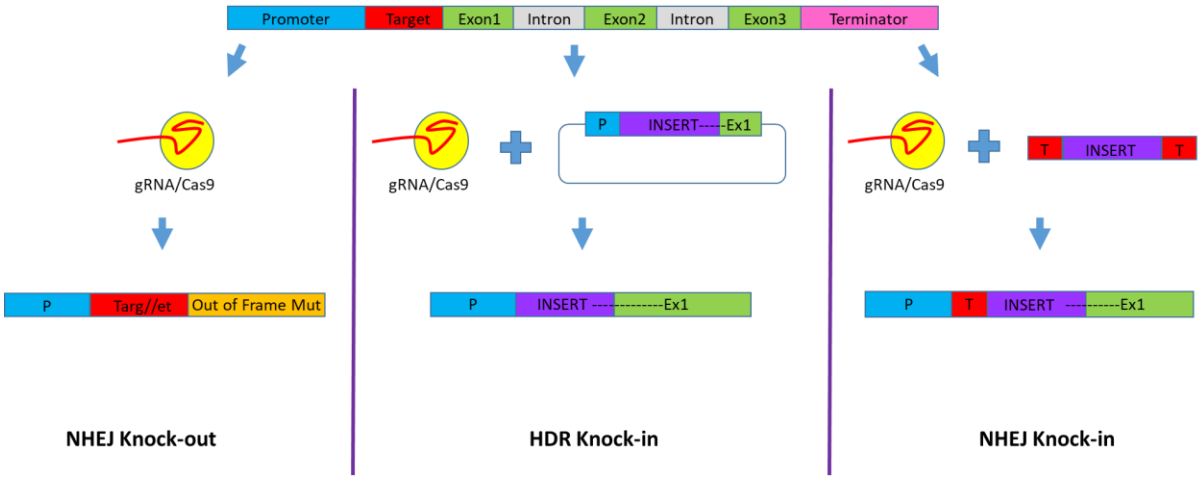

Figure 1.5 Common repair mechanism for inducing CRISPR mediated gene knock-in or knock-out after induction of double strand breaks by Cas9 nuclease

After Cas9 mediated DNA cleavage, the two blunt ends of the target sequence are normally quickly repaired by the cell. This normally occurs via the error prone non-homologous end joining pathway (NHEJ) which is error prone and often induces indels. If this occurs within a coding region this can lead to frame shifts downstream and induce loss of function. This is how most CRISPR mediated knock-outs occur. The homology dependent repair pathway is used less frequently but uses homologous sequences in nearby DNA as a template to repair the site of nuclease cleavage. It is a useful method for integrate new genetic sequences at cut sites and results in a repair with high fidelity to the homology donor. Non-homology mediated end joining can occur when blunt ends from donor vectors and the target site are ligated via NHEJ, although there is no control over the orientation of the inserted donor.

1.4 Oogenesis, sex determination and early embryogenesis in insects

Insects differ vastly from vertebrate systems in their early development which has implications when developing or adapting transgenesis techniques to these systems. Reproductive mechanisms and development patterns also vary greatly between and within orders of insects themselves and so it should not be taken for granted that methodologies are instantly transferable.

1.4.1 Oogenesis

Gamete production, like vertebrates, occurs in the insect sex organs with oogenesis happening within the adult female ovary and spermatogenesis within the testes. These organs originate from germ cells, which are specialist reproductive precursor cells that originate very early in development and can either be maternally transmitted as germ plasm to the embryo (as seen in Zebrafish and *Xenopus*) or be zygotically induced during embryogenesis (as in mammals). The germline specification strategy is not conserved between orders and appears to vary between individual species, but a conserved gene *oskar* is essential and sufficient to specify germ plasm in species that have maternal transmission (some species in *Diptera, Hymenoptera, Coleoptera*)[243]. The exact methodology by which zygotic induction of germ tissue occurs, differs amongst insect orders. In *Gryllus bimaculatus*, bone morphogenetic protein (BMP) signalling, along with Blimp-1 activity downstream has been shown to be important in inducing germ plasm although it is not currently known exactly how species such as *Apis mellifera* or *Tribolium castanateum* specify their germ plasm[243]. In *Lepidoptera* it is not clear whether a germ plasm is maternally transmitted or induced. Germ cells only appear after germ band formation, however

nanos O mRNA granules are maternally deposited during oogenesis and correlate loosely with germ cell location in *Bombyx* and *Pararge*[199,244].

Oogenesis in insect occurs in string like ovarioles within the ovary, with each oocyte surrounded by follicle cells. The method by which oocytes mature again varies, with broadly two types of oogenesis described that are influenced by the type of ovariole and can influence the germ band type and polarity of the embryo[163].

Panoistic oogenesis has a simpler morphology, where the oocyte is surrounded by follicle cells but have no internal specialised supportive cell subtypes and is seen in Orthoptera, Apterygota, Ephemirida and Siphonaptera[164]. The most studied form of oogenesis is meroistic oogenesis which is seen in most other insects. Here specialised trophic cells support the development of the oocyte. There are subtypes of meroistic oogenesis, with telotrophic ovariales (such as seen in Hemiptera and Coleoptera) linked to and aided by a cord of trophic cells which breaks off in late oogenesis, and polytrophic ovarioles (as seen in Diptera and Lepidoptera) in which both nurse cells and the oocyte are surrounded by the follicular layer with nutrients flowing into the oocyte through gaps in the nurse cell membranes[164]. The nurse cells and oocyte themselves are produced from a single cystoblast cell which undergoes multiple rounds of incomplete mitotic division. The exact number of number of divisions varies depending on the insect species[165] but only one of these will undergo meiosis to form an oocyte with the rest becoming nurse cells. The nurse cells will then undergo substantial DNA replication via endomitosis, vastly increasing their DNA content.

The next stage of oogenesis is vitellogenesis, where nurse cells take up vitellogens proteins from the haemolymph and process them to form vitellins which constitute

the yolk. This provides energy for the developing embryo. The nurse cells are also responsible for transcribing a large volume of maternal ribosomal and messenger RNAs as well as proteins that drive early development and determine embryonic fate. Eventually the nurse cells break down and are absorbed into the cytoplasm after which follicular cells then secrete a vitellogenic and chorionic layer on the outside of the oocyte which protect the embryo from desiccation or damage[166].

Not all insects are exclusively oviparous though, some such as species such as members of the Aphididae family alternate between viviparous and oviparous seasonally. Maternal factors present in the ovariole during spring and autumn cause viviparous oocytes to develop differently and produce diploid oocytes. The mechanism by which these diploid oocytes are formed is unclear, with both mitotic and incomplete meiotic (endomeiotic) divisions reported in aphid species [167,168], however these oocytes develop lacking a chorion or vitelline membrane, and yolk[169]. Nutrients are instead absorbed directly from the maternal haemolymph into the substantially smaller oocyte[170]. Embryogenesis is temperature dependent but occurs much more rapidly than with oviparous embryos designed to overwinter, requiring only a fraction of the time to reach the nymph stage [171].

After oogenesis, fertilisation may then be required to successfully produce offspring but this does not apply to all systems. Variations in sex determination, sex chromosome types and reproductive systems and life histories can result in fertile individuals developing from haploid, diploid or unfertilised embryos without the involvement of a male gamete.

1.4.2 Sex determination

Most insects will have a sexual reproductive stage where gametes will be exchanged between males and females resulting in fertile offspring with genetic elements from both parents. This is not always the case, as parthenogenesis makes up a major part of the reproductive life histories of both Aphididae, as previously discussed, and Hymenoptera.

There are four common types of ways that sex is determined in insects[172]. Male heterogamy (such as XY/XX in most Diptera and Coleoptera) or female heterogamy (such as ZZ/WZ in Lepidoptera) involves diploid males and females possessing separate sex chromosomes, with a male gamete in the form of sperm required to fertilise the oocyte to produce a zygote. Haplodiploidy, where males hatch from unfertilised eggs and females from fertilised ones is most widely studied in Hymenoptera, with all males in social insects species such as bees, wasps ants female are haploid and females diploid. Paternal genome elimination occurs in Coccoidea (scale insects)[173] and possibly certain Coleoptera[174], where the paternal transmitted genome chromosomes are inactivated as heterochromatin and eliminated during spermatogenesis so only the maternal chromosomes are transmitted.

In heterogamous organisms like *Galleria* sperm is transferred to the female during mating, and stored in a specialised organ called a spermatheca. This is then used to fertilise the mature oocyte via a specialised fertilisation channel in the chorion, called the micropyle, that attracts the sperm[175]. The fertilised embryo is then normally laid immediately or retained until a suitable oviposition substrate is found by the female. Unless eggs are retained this normally means that the two pronuclei have not yet

fused at the time of laying, with the oocyte undergoing its second meiotic division after arrest during meiosis I during oogenesis[176].

1.4.3 Blastoderm formation

After meiosis II, one of the 4 maternal haploid meiotic products undergoes fusion with the spermatozoa pronucleus, with the other three forming polar bodies. In Lepidoptera the fates of the polar bodies are unclear but it has suggested they are able to fuse with supernumerary spermatozoa pronuclei[3,177].

In all studied insects the zygotic nucleus then undergoes a serious of rapid and continuous endomitotic divisions, with the daughter nuclei emigrating to the cell periphery as energids. These divisions are not driven by endogenously produced proteins from genomic expression and instead recruit maternally provided mRNAs and proteins stored within the cytoplasm. What occurs when these energids reach the cytoplasm the periphery depends on the species of insect. Some, such as *Drosophila* and *Tribolium*, continue to undergo multiple rounds of incomplete mitotic divisions at the cell cortex with all nuclei contained within the same cytoplasm but small membranous invaginations known as furrows maintaining spatial localisation [178,179]. During these rounds of syncytial divisions in *Drosophila*, a subset of nuclei at the posterior pole cellularise before others– these are the pole cells which go on to form the germ line tissue. These rounds of syncytial division on the cortex are not found in all insects though with lepidopteran and orthopteran energids reaching the cortex at different times and cellularising without subsequent divisions [180,181].

In insects it is generally assumed that cellularisation of energids to form the blastoderm marks the onset of zygotic gene expression although the exact mechanism of gene repression up to that point is not known. Two theories have

been posited to explain the phenomenon[182], although they may not be mutually exclusive. The first is that large numbers of repressing factors present in the cytoplasm are able restrict gene expression until titrated down by increased nuclear mass within the embryo. The second is that a biochemical cascade early in embryogenesis sets a molecular clock that represses gene activation up to a certain point. What is known is that in *Drosophila* zygotic expression seems to be activated by the maternally encoded transcription factor Zelda which binds to a large number of heptameric targets within the genome, inducing expression[183].

1.4.4 Germ formation

After blastoderm formation, embryonic cells differentiate into embryonic and extra-embryonic tissue which will go on to form the serosal layer. The mode by which the embryonic tissue, known as the germ anlage, forms and later developments has led to 3 main patterns being described – short, intermediate and long germ embryogenesis (Fig 1.6).

Generally understood to refer to the size of the germ band in relation to the size of the embryo, these actually refer to the number of segments specified pregastulation [184], with long germ insects having almost all segments specified. The size and localisation of the nascent germ band varies greatly between insect; in some species, such as *Drosophila*, it can take up the entire length of the embryo, whereas in members of Orthoptera it can cover only a small fraction. It is also dynamic and can expand or contract during development. The size and location of germ bands within insect embryos has implications for transgenic technologies that rely on gene translation to be active. Methodologies which rely on DNA encoded transposases or nucleases require injection material to be incorporated into germ tissue at

cellularisation and thus must be injected at the embryonic location where these future cells will form.

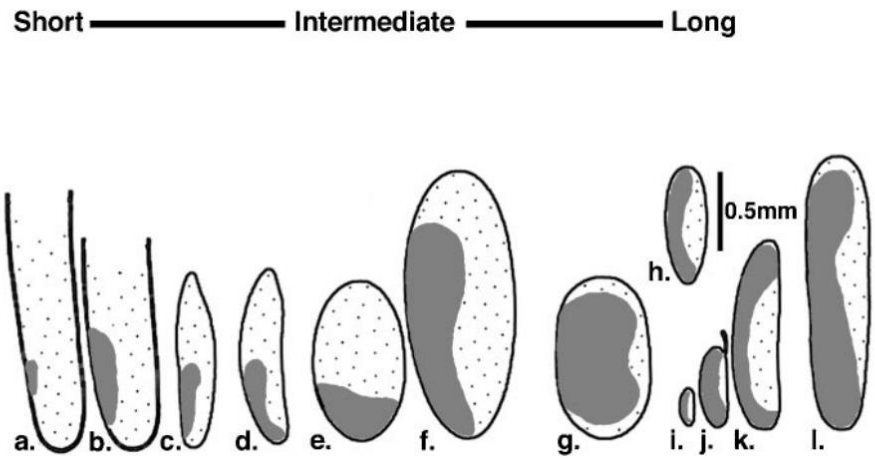

Figure 1.6 Germ types in embryos of different insect species

Insect eggs of various germ types, adapted from Davis and Patel[184]. Insect eggs are to scale and the germ anlage is represented by the shaded grey. Orthoptera: a, *Oecanthus pellucens*; b, *Acheta domesticus*. Odonata: c, *Platycnemis pennipes*. Hemiptera: d, *Euscelis plebejus*. Coleoptera: e, Atrachya menetriesi; f, *Leptinotarsa decemlineata*; h, *Bruchidius obtectus*. Lepidoptera: g, *Bombyx mori*. Diptera: i, *Smittia sp.*; j, *Drosophila melanogaster*; k, *Calliphora erythrocephala*. Hymenoptera: l, *Apis mellifera*.

1.4.5 The mitotic spindle

Development from adult cell progenitor cells into gametes and then zygotic tissue is based on the ability of cells to replicate and divide. In particular, early insect embryogenesis is defined by multiple sets of synchronised mitotic divisions. These divisions involve major restructuring and segregation of cellular components and chromosomal material by the cytoskeleton. A key component of this cytoskeletal system is the microtubule network, a fibrous and dynamic scaffold structure that plays a key role in intracellular vesicle and organelle transport and a vital part of replicative machinery, forming meiotic and mitotic spindles[185].

Microtubules themselves consist of bundles of protofilaments, helically arranged to form a tubular structure roughly 25nm in diameter. The protofilaments themselves are polymers made of heterodimers of α and β-tubulin which can be added (polymerised) in a guanosine triphosphate (GTP) mediated fashion or depolymerised to grow or shrink the microtubule[186]. Due to the consistent orientation of the α and β-tubulin molecules within the strand, the microtubules have a polarity with the α-tubulin led and faster growing end labelled the plus end and the β-tubulin led and slower growing end the minus. The ability to polymerise and depolymerise in a co-ordinated and controllable fashion is vital to microtubule function within the cell and issues with this system can lead to cancerous or neurodevelopmental pathologies in humans [187,188].

The alignment, orientation and separation of chromatids is mediated via microtubules and the proteins associated with them during mitosis. In rapidly proliferating cells, microtubules are primarily originated from a centrosome which act microtubule organising centres. Centrosomes contain a pair of centrioles, which use

a γ-tubulin ring complex complementary to α/β tubulin heterodimers as a template to nucleate new microtubules. During mitosis, and after nuclear envelope breakdown, large, branching networks of microtubule fibres enter the nuclear space from centrosomes on either side to form a dense structure known as a spindle[189]. Motor proteins such as dynein and kinesin, and other microtubule associated proteins (MAPs), are able to mediate kinetochores binding and generate the necessary tension to align sister chromatids on the equatorial plate. After checks to ensure correct chromatid alignment, they are then involved in dragging each sister chromatids apart and thus correct segregation of nuclear material. Key to the robustness of the spindle is the level of branching, which is primarily mediated by the augmin complex [190–192]. This MAP is thought to enable branching via providing an anchor for γ-tubulin ring complexes to bind at an angle and allow new microtubules to form at an angle.

Little is known about spindle formation or morphology during mitosis in insects outside of *Drosophila*. Large differences in chromosome number and types of kinetochore attachments between orders would likely cause observable changes in spindle morphology as described by Wolf[193–196], however no investigations to date have studied how microtubule dynamics and MAPs are involved.

Aims

The specific aims of this project were to:

- **Develop and optimise methodologies for microinjection in *Galleria* embryos**
    - Optimise a *Galleria* rearing protocol to produce large numbers of age defined embryos
    - Investigate the timing and pattern of early mitotic divisions within *Galleria* up to blastoderm formation
    - Develop and optimise a protocol for introducing foreign material into *Galleria* embryos via microinjection
- **Develop the first transgenic *Galleria***
    - Utilising existing transgenic techniques, integrate a fluorescent reporter into the genome to generate the first transgenic *Galleria* strain
    - Optimise this methodology and introduce more complex transgenes to create strains with a visible subcellular mitotic phenotype.
    - Utilise newly created strains to attempt a gene knock-out with visible phenotype using CRISPR mediated mutagenesis

Delivery of these main aims will provide a much broader range of applications for this model organism. The ability to successfully inject embryos will open techniques beyond just the creation of transgenics and enable more robust studies into the development of this organism. *Galleria* will also become a much more desirable replacement for rodent models once genetically tractable which could have large implications on the goals of replacement, reduction and refinement of animals in research (3Rs).

Chapter 2

Materials and methods

2.1 Animals and husbandry

An inbred “wild type” *Galleria mellonella* colony was started, with the primary purpose of being able to consistently collect large numbers of embryos within short (hourly) time periods. Tru-Larv late stage larvae (Biosystems technology) were fed on an artificial honey diet (Diet 3, Jorjao et al. 2018[5]) at 30C, constant darkness. 50-75 late-stage larvae/pupae were transferred to clear PET sweetie jars containing a small amount of diet and allowed to hatch and mate. Embryos were collected for colony maintenance from folded wax paper secured with a paper clip at one end that had been inserted into the adult jars. These embryos were then placed directly onto artificial honey diet and allowed to develop in opaque PET larval jars, with 5um steel mesh between the lid and the jars to reduce L1/L2 larvae escape, until last instar/pupation whereupon they were transferred to PET sweetie jars as above. The number of larval and adult jars put down per week can be varied depending on the number of experimental animals needed and the life stage required, but at least one larval and adult jar per week ensures a regular turn-over and easy ability to expand the colony. This colony has been under continuous culture from 2018. More detailed protocols can be found in supplemental material 1.

2.2 Embryo Dechorionation

Embryos were immersed in a 1.125% sodium hypochlorite solution and agitated until both separated and their chorions removed. The embryos were then strained into a dechorionation basket made from a modified 50ml falcon tube with the bottom conical part removed and a hole cut in the screw cap, the screw cap holding in place some nylon mesh. The embryos were briefly rinsed with embryo wash (0.05% triton x 100 solution) and immersed in tap water for 30 seconds. The dechorionation basket was then held under a gentle stream of tap water, which ran across the sides and bottom of the mesh until droplets dripping off the basket would no longer discolour blue roll to a pink colour.

2.3 Embryo fixation, staining and immunohistochemistry

Embryos were fixed by transferring to a 15ml falcon tube containing 5ml of 1:1 ratio of heptane and methanol, using a paint brush. The falcon tube was gently inverted, and embryos that fell to the bottom methanol fraction were gently aspirated into a fresh tube using a Pasteur pipette. These embryos were washed twice further in fresh methanol and stored at 4C overnight.

Embryos were rehydrated in 3x15 min washes in PBS + 0.1% Triton x100 (PBSTr). Embryos were then blocked in PBSTr + 3% BSA for 30 mins before adding the primary antibodies to the solution and incubating at 4C with gentle rocking overnight. Antibodies used were a custom anti – *Dm* Pnut (Biorad, 1:500) from the Wakefield lab and anti –tubulin DM1A+DM1B mouse (Abcam ab44928, 1:2000). A further washing with 3 x 5 min washes in PBSTr was performed before incubating with the corresponding secondary antibody (Abcam goat anti-rabbit ab150077 and Abcam goat anti-mouse ab150113) at RT for an hour. The embryos were then stained with

0.5mg/ml Hoechst 33258 in Hoechst buffer for 20mins at room temperature, before a final 3 x 5 min washes in PBS + 0.1% Triton x100.

2.4 Imaging

Embryos were mounted on slides within 2 stacked ring binder reinforcement sitckers (Rymans) in Vectashield (Vectalabs) mounting media before covering with a cover slip and securing with nail polish. They were imaged using a Nikon TE-2000s inverted microscope. Larvae were imaged under a Leica MZ10F fluorescence stereomicroscope with a GXCAM HiChrom-HR4 HiSens camera.

2.5 Microinjection

Tubs of mixed sex adult moths were allowed to lay in total darkness for 1hr after which embryos were collected (embryos are 0-1hr old). These embryos were aged for a further hour at 30C, before dechorionation. Embryos were then aligned on glass slides using a paint brush trimmed to a single hair, either kept in place by hydrostatic tension or backed onto a coverslip glued in the centre. They were aligned in a rough “head to tail” orientation with the flatter surface kept parallel to the slide where either set of characteristics could be determined. The embryos were then placed in a humidified chamber until needed for injection.

Microinjection solutions were loaded into FemtoTips (Eppendorf) and injected with a FemtoJet + InjectMan microinjection/manipulation system mounted to a Nikon TE2000s inverted fluorescence microscope. Embryos were injected with a droplet diameter roughly corresponding to 1/5th of the total embryo diameter.

2.6 Plasmids

The plasmids pHA3PIG [109], pPIGA3GFP [109] and pBachsp90GFP-3xP3DsRed [204] were kindly provided by Prof. Hideki Sezutsu of the National Institute of Agrobiological Sciences (NARO), Tsukuba, Japan.

The helper plasmid pBmhsp90hyPB was generated using the hyper-active PiggyBac transposase from the expression vector SPB-DNA (Hera Biolabs), under the control of the *Bombyx mori* hsp90 2.9kb fragment from pBachsp90GFP-3xP3DsRed and the wild type PiggyBac transposase 3' UTR from pHA3PIG and inserted int0 the kasI/smaI digested backbone of pHA3PIG via Gibson assembly using an NEBuilder kit (NEB).

The expression vector pGmhsp90:GFP was generated using EGFP with an added short c terminal Thr-Gly-Gly-Thr linker containing AgeI and KpnI sites under the control of the 2kb upstream region of *Galleria mellonella* hsp83 (hsp90) and the SV40 polyA terminator inserted between the donor sequences of AscI/XhoI digested pBachsp90GFP-3xP3dsRed via Gibson assembly. pGmhsp90:GFP-αtub1b was generated by sequential digestion of pGmhsp90:GFP with AgeI and KpnI and inserting the *Galleria* α tubulin 1b cDNA sequence also via Gibson.

The expression vector pBmhsp90:Histone2AV-mCh;P3DsRed was generated by digesting the pBachsp90GFP-3xP3DsRed plasmid with PmlI and AscI before ligating in a synthesised fragment containing G.*mellonella* Histone 2AV connected to MCherry via a short linker and with a SV40 poly A terminator via Gibson assembly.

All plasmids were propagated in NEB 10β cells and midiprepped using Nucleobond Xtra midi kits (Machery-Nagel) and sequenced using Eurofins short read sanger sequencing across the junctions of insertions.

Plasmid Name	Description	Provider	Reference
PB531A-2 (PB-EF1-MCS-IRES-RFP)	EF-1α driven RFP expression	Cambridge Bioscience (Produced by system biosciences)	https://www.bioscience.co.uk/product~533384
PB513B-1 (PB-CMV-MCS-EF1-GreenPuro)	EF-1α driven GFP expression + puromycin	Cambridge Bioscience (Produced by system biosciences)	https://www.bioscience.co.uk/product~375782
pBachsp90GFP-3xP3DsRed	*Bombyx mori* heat shock protein 83 (hsp90) 3kb upstream region driving EGFP expression + 3xP3 eyeless artificial neuronal/eye specific promoter driving RFP To avoid confusion with other hsp90 plasmids in this manuscript, this is referred to as: p*Bm*hsp90:GFP/3xP3:DsRed	Hideki Sezutsu Japanese Institute of Agrobiological Sciences, NARO	[204]
pPIGA3GFP	*Bombyx mori* cytoplasmic actin(A3) upstream region driving GFP expression	Hideki Sezutsu Japanese Institute of Agrobiological Sciences, NARO	[77]
pBac[Pub-nls-EGFP]	*Drosophila melanogaster* polyubiquitin promoter driving the expression of EGFP with a nuclear location signal	Marc Schetelig University of Geissen	[206]
pHA3PIG	*Bombyx mori* cytoplasmic actin(A3) upstream region driving *T. ni* PiggyBac transposase	Hideki Sezutsu Japanese Institute of Agrobiological Sciences, NARO	[77]
PB210PA-1	CMV driven Super PiggyBac Transposase expression vector	System biosciences/Hera biolabs	https://www.systembio.com/super-PiggyBac-transposase-expression-vector

Table 2.1: PiggyBac Donor and Helper Plasmids obtained through Innovate UK grant before the start of this project

Primer Name	Sequence	Product length
TubA1a Fwd1	ATGGTGGACAACGAAGCCAT	266
TubA1a Rev1	ATCTCCGCCACAGACAACTG	
TubA1a Fwd2	CAGTTGTCTGTGGCGGAGAT	208
TubA1a Rev2	CCACTTTGAACCCAGTCGGA	
TubA1b Fwd1	TAGTACTCGACCGCATTCGC	255
TubA1b Rev1	AAGCACAGTCAGAGTGCTCC	
TubA1b Fwd2	AACGTCCGACCTACACCAAC	173
TubA1b Rev2	GGAGATGACTGGGGCGTATG	
TubA2 Fwd1	GTATCTCCGTCCACATCGGC	215
TubA2 Rev1	CACAACCGTAGGCTCCAAGT	
TubA2 Fwd2	TCCAACCTGATGGGCAGATG	285
TubA2 Rev2	GCTTCCTGATCCTGTCGAGG	
TubA3 Fwd1	CTGGGGAAAACTCGACCACA	169
TubA3 Rev1	CTGCATGTCCGATGTCTCCA	
TubA3 Fwd1	TGGATTGGTGTCCAACAGGG	298
TubA3 Rev2	TGCATGTCCGATGTCTCCAT	
TubA1b CDS Fwd	CTCAATTCAAAATGCGTGAGTGCATC	1376
TubA1b CDS Rev	CGCCTTACGCGTTTAATATTCTTCTGC	
His2AV Fwd1	ATGGTCGTGTTGGAGCTACG	143
His2AV Rev1	GATGGCAAGCTGCAAGTGAC	
His2AV Fwd2	TGTATCCAGATCAGCGCGAG	114
His2AV Rev2	GAATAAACAGCCGCCGTAGC	
GFP Fwd	CTGGTCGAGCTGGACGGCGACG	630
GFP Rev	CACGAACTCCAGCAGGACCATG	
iPCR5' Fwd	ACCGCGTGAGTCAAAATGACG	
iPCR5' Rev	CCAAGCGGCGACTGAGATGT	
iPCR3' Fwd	CAGACCGATAAAACACATGCGTCA	
iPCR5' Rev	TGGACAAACCACAACTAGAATGCAG	

Table 2.2 PCR primers

2.7 Nucleic acid purification and extraction

Genomic DNA was extracted using the Monarch gDNA extraction kit, following the insect tissue protocol. gDNA for screening crispr knockouts was extracted by homogenising a single embryo with a pipette tip and then heating at 97C in 90ul 50mM NaOH for 20 mins before neutralising with 10ul 0.5M TRIS-HCl. For larval screening, whole larvae were frozen in liquid nitrogen before homogenising all or part of the larvae in an Eppendorf and adding 100ul squish buffer (10 mM Tris – pH 8.2, 1 mM EDTA, 25 mM NaCl) + proteinase K.

Plasmid DNA was purified using either NEB Monarch or Thermofisher Genejet miniprep kits, and for larger preps Macherey-Nagel's NucleoBond Xtra Midiprep kit was used.

Total RNA was extracted from homogenized embryos or tissue by adding roughly 10x vol of TRIzol reagent, followed by triteration and incubation at RT for 5 mins. Chloroform was added to the mixture at 20% of the TRIzol volume, and inverted 3 times before incubating at RT for 3 mins. After spinning at 12k g for 15mins at 4C, the aqueous phase was transferred to a fresh Eppendorf before adding roughly 50% isopropanol by volume, mixing and incubating at RT for 10mins. This was then spun at 12k g at 4C for 10 mins, the supernatant removed and the pellet washed twice with cold 75% ethanol. The pellet was then air dried, resuspended in nuclease free water and quantified. cDNA was synthesised from total RNA using Applied Biosystem's High Capacity cDNA Reverse Transcription kit and either random hexamers or an anchored Oligo-DT (20) primer (IDT) according to the manufacturer's instructions.

2.8 PCR screening

A ~600bp portion of the GFP coding sequence was amplified using KOD hot-start proof-reading Taq polymerase (Toyobo) using primers GFP F and GFP R. PCR fragments were sequenced using Eurofins short read sanger sequencing across the junctions of insertions.

2.9 Inverse PCR (iPCR)

Genomic DNA was digested for 2hrs with MspI, heat inactivated and then re-ligated overnight at 4C to promote circularisation. PCR was then performed using primer pairs iPCR5' F & R and iPCR3' F & R .iPCR fragments were sequenced using Eurofins short read sanger sequencing across the junctions of insertions.

2.10 Transgenic animal screening and line maintenance

Immediately post injection, embryo slides were transferred to humidified petri dishes and incubated at 30C for 4 days. At day 5 these petri dishes were then placed into larval rearing tubs containing diet, with the petri lids wedged open slightly to allow easier larval escape to the diet and prevent excess humidity prevent successful hatching. G0 Larvae were allowed to develop until roughly 1cm long and then screened for fluorescence using either an Olympus SZX16 or Leica MZ10F fluorescence microscope with both GFP and DsRed filter sets. Mosaic fluorescent larvae were separated from non-fluorescent G0s and reared separately. G0 injected adults were mated either back to wild type adults or to siblings, and the G1 progeny screened for fluorescence at both embryonic and larval stages.

For maintenance of non-WT strains a slightly different rearing protocol was used to wild types, with embryos transferred to a small amount of diet in urine sample pots and closed with cotton flugs at 30C, 12hr light/dark. Once larvae reached around

1.5cm long they were screened for phenotype and transferred to fresh diet in petri dishes until pupation. Pupae were sexed by on abdominal morphology and could then be placed in separate petri dishes at 25C with egg papers and the adults either sibling mated or crossed to wild type.

2.11 Guide RNA design and ribonucleoprotein complex assembly

Alt-R CrRNAs and Tracr-RNAs were purchased from IDT, with CRISPOR (Concordet and Haeussler 2018) used to estimate cutting score and off-target effects. These were then duplexed according to manufacturer's instructions. A GFP sgRNA described by Jao et al. 2013[197] complementary to the sequence GGGCACGGGCAGCTTGCCGG was in vitro transcribed using the protocol previously described by Burgher et al. 2016[198].

sgRNA or Cr/Tracr RNA duplexes were then in vitro assembled into RNPs with Cas9p-NLS-MCh as previously described[198] and kept on wet ice until loading into microinjection needles.

2.12 Tubulin Phylogeny

Tubulin phylogeny was obtained using full length CDS for *Galleria* α-tubulins available on NCBI and by blasting *Drosophila melanogaster* α-tubulins against the *Galleria* proteins. These full and partial proteins were then manually mapped back onto the Honeycombmoth V1 assembly and the full CDS identified. CDS for *Galleria* tubulins used are available in supplemental material 2. Phylograms were assembled using the phylogeny.fr web service[241] using T-Coffee multiple alignment and

MrBayes 3.2 Bayesian inference (1000 generations/sample every 100) to construct the tree.

2.13 Tissue sample preparation

Larval tissues were flash frozen in liquid nitrogen. Small tissue sections were cut off and then homogenised as much as possible on ice in a 1.5ml eppendorf tube using a micro-homogeniser before adding equal volume of RIPA buffer in µl (abcam) to the weight in µg of tissue and homogenising for a further minute. Samples were then spun at 5000g for 5 mins at 4C, before removing the middle layer and adding 2x laemmli buffer. Samples were boiled for 10 mins before storing at -20C.

2.14 SDS-PAGE and Western blot analysis

Proteins samples were separated using on 10% acrylamide gels using SDS-PAGE and wet transferred onto nitrocellulose membranes. These were probed with rabbit anti-GFP (abcam ab290) or rabbit anti-dsRed (abcam ab62341) primary antibodies of 1:500 at the manufacturers recommended concentrations and incubated overnight at 4C. IRDye 800CW goat anti-rabbit and (LI-COR) IgG polyclonal antibodies were used for secondary detection. The blots were then imaged using an Odyssey CLX system.

Chapter 3

Development of a microinjection pipeline for *Galleria*

3.1 Introduction

An obstacle to uptake of *Galleria*, and a limit to its potential as a replacement for rodent models has been the lack of a molecular genetic toolkit with which to engineer the organism. The most common method for generating transgenic insects and other model animals, is to inject a small amount of mutagenic substance such as DNA, protein or a combination thereof into the zygote with microneedles, resulting in the desired modification to the genome. This method of delivery, termed microinjection, is a versatile way of introducing other exogenous substances too, enabling techniques such as RNAi or transient expression of fluorescently tagged proteins of interest.

Insect embryos possess a tough serosa known as the chorion, deposited during oogenesis to protect them from desiccation and damage in the environment as they develop. This layer can present a challenge to microinjection as the delicate needles used must penetrate it to deliver the desired cargo inside the embryo, often causing damage to either the needle or the embryo itself. To circumvent this, microinjection protocols for some insect species with particularly hard chorions have used methods that either first introduce a small hole using a tungsten needle through which you can inject or remove the outer chorionic layer entirely. With both methods, the embryos are vulnerable to desiccation afterwards, unless care is taken to maintain humidity levels in the post injection environment.

Galleria embryos are laid in clusters by the adult female, who uses her long ovipositor to deposit them in crevices within their preferred substrate. The individual embryos vary in shape between elliptical and round, measuring around 0.5mm in diameter and are glued to both other embryos from the clutch and the substrate by a secretion from the mother[2,6,13]. Whilst soft when first laid, this outer chorionic layer quickly hardens and is impenetrable by standard microinjection needles.

Whilst being able to penetrate the chorion is important, equally important is to ensure that mutagenic mixtures are injected at the correct time during development. The best time to inject is normally when the embryo is at the single cell stage. From what is known about the early development in other Lepidoptera and insects, meiosis II and fertilisation often occurs post-oviposition[176]. The zygotic nucleus then completes multiple rapid but incomplete mitotic divisions, with the sister nuclei migrating to the periphery of the embryo. During this period the migrating nuclei (energids) are within a common cytoplasm and form a multinuclear single cell known as a syncytium.

In *Drosophila* these nuclei undergo several rounds of synchronous divisions once they reach the periphery, except for those at the posterior pole of the organism which form the future germ cells. During these rounds of nuclear division at the periphery the embryo is said to be a syncytial, until it cellularises to form a cellular blastoderm. Development up to this point is almost entirely driven by translation of maternal mRNA transcripts with genomic transcription machinery predominantly activated only at cellularisation (the maternal-zygotic transition). In *Bombyx mori*, the lepidopteran model, this process is slightly different as reported by Nagy et al[180]. Energids cellularise immediately upon reaching the periphery, with those at the anterior arriving first. The cellular blastoderm then quickly differentiates into germ anlage, serosal and yolk nuclei populations. The future germ cells originate from a small

population within the germ anlage, located towards the posterioventral surface of the embryo[199]. It has not been confirmed to what extent zygotic transcription occurs during syncytial development in lepidoptera, but maternal transcripts are presumed to be the primary drivers of development up to cellularisation.

The importance of this is that certain transgenic techniques, such as PiggyBac transformation, often involve the expression of DNA-based helper plasmids. Due to the lack of transcription these are not likely to be active during the initial development of the organism but still must be injected before cellularisation in order to be present in the cytoplasm and taken up into the cells. It is advantageous to inject them in regions that will go on to form germ cells if they are to generate integrations that will be passed on to the next generation.

In this chapter describe how I developed and optimised procedures for maintaining a *Galleria* colony in order to consistently produce large quantities of embryos and protocols that enabled robust separation and dechorionation of early (<2hr old) *Galleria* embryos. I further developed a protocol enabling microinjection of exogenous substances and demonstrate its efficacy in maintaining viable *Galleria* throughout embryonic development following microinjection. In addition I undertook a an analysis of early development in the *Galleria* embryo, which determined the timings for transition from first division to blastoderm formation.

3.2 Establishment of a *Galleria* colony for microinjection

Whilst several methods for rearing *Galleria* exist[5,6,60,200], there is no standardised protocol, and most are designed to provide large numbers of larvae. This project would require large numbers of embryos at an early stage and so I sought to develop

a protocol that produced the desired life stages in sufficient quantity and was easy enough for a single researcher to use.

It has been reported that *Galleria* do not feed after emergence from the pupal stage, instead surviving on reserves built during their larval stage[201]. It is known in other species that the lifetime fecundity of insects tends to correspond to their adult size，which in turn corresponds with the size of their last larval instar before pupation. With that in mind, I decided that the best way to ensure highly fecund adults would be to rear the larvae at a low density, which should promote quicker larval development times and larger larvae. Reports from the literature suggested that 0.75g of diet per larvae was sufficient for development to pupation [60], and so this was used as a starting point. This required some way to know the number of embryos seeded but due to their very low weight, measuring the mass of single embryo clusters tended to be an imprecise way to estimate embryo number. Instead after counting the numbers of embryos in the first few clusters is used, I was able to estimate roughly the numbers laid.

During the early stages of rearing the colony, I found the seeding density to be too high, as larvae would eat all their diet before reaching their final instar. This often resulted in smaller larvae that took longer to develop and resulted in mould growth swiftly forming on frass, as humidity built up. In addition, it also increased the complexity of the manual maintenance of the moths, with regular checking of larvae, splitting populations and replenishing diet.

Figure 3.1: Wild type rearing colony inside incubator

Wild type *Galleria* colony with adult jars seen on the second shelf from top, and larval jars third from top. Blue roll and bait dishes containing larval diet are placed on each shelf to attract any L1 larvae that have managed to escape through the lid/mesh/jar interface.

Seeding larvae at a lower density of approximately 1g of diet per larvae resolved most of these issues, and allowed uninterrupted development in a single jar from embryonic stage to last instar larvae, with ~150 embryos normally producing around 100 larvae.

Upon the onset of pupation in larval tubs, 50-75 late stage larvae/pupae were transferred to clear PET sweetie jars containing a small amount of diet and allowed to hatch and mate. More larvae could be used, but I found when greater numbers were added to the adult jars humidity build up once again became an issue. The adult moths were also much more likely to disturb each other when reared in higher density, increasing the rate at which they lost their flight scales and impacting their behaviour. Since they were more prone to running around the jars and disturbing other adults which might be laying, counterintuitively I found that I got slightly bigger and more consistent embryo yields from slightly smaller numbers of adults. The adults oviposit on most surfaces in the jars but have a particular preference for tight crevices or ridges such as those found in and behind pupal casings. Embryos laid there and not on the egg papers are normally useless as removing them from their substrate normally destroys them. To maximise embryo yield, I recommend removing pupae or casings that are present on the underside of the lid or the sides of the adult jars and place them at the bottom. Adding a small amount of egg carton to the bottom when setting up adult jars can promote pupation there rather than on other surfaces.

The focus on maximal embryo production allowed large numbers of staged embryos to be acquired, which was vital for this project. However, the methodology can also produce large numbers of size matched, healthy larvae that are suitable for infection studies with minimal extra effort for the researcher.

3.3 *Galleria* Microinjection Pipeline

3.3.1 Embryo Separation

The hard chorionic layer and strong adherence of embryos to their neighbours and the oviposition substrate presented a challenge for injection. To try and rectify this I continued work by Amy Housden, a research technician who was working on a short-term Innovate UK grant jointly held by Prof. James Wakefield and Biosystems Technology, looking at whether it would be possible to penetrate through the chorion.

I found that in the first 15 minutes post oviposition (PO), the chorion of *Galleria* embryos is very soft. However, embryos are laid in clusters and attempts to remove individual embryos at that early stage often led to complete embryonic collapse. Trying to inject embryos at this 0-15min stage was technically difficult as the short time window and clustered nature of the embryos meant that it was often impossible to inject all the embryos in a cluster using the available equipment. The short oviposition period also reduced the embryo yield, as I found that I frequently failed to collect any embryos as the moths take a short while to start laying again after being exposed to light. I concluded that injecting the very early embryo through the chorion was not likely to be a technique that would work on the scale needed to generate transgenic strains.

I therefore experimented with a variety of treatments on young (1-2hr) embryos. At 1 hour PO, the chorion had hardened to an extent that attempting to inject through it often resulted in a needle breakage or required such a stout needle that the embryo was destroyed. TI therefore investigated ways to both separate individual embryos from egg clusters and to soften or remove the chorions to allow microinjection (Table

Treatment	Embryo Age	Dechorionation Agent Concentration	Dechorionation Agent Time	Effectiveness of Embryo Separation and Dechorionation
Benzalkonium Chloride	1-2hrs	5%	90s	No effect
NaOH	1-2hrs	0.25M	5-10 mins	Moderate separation
Bleach + Triton X-100	1-2hrs	5% active chlorine + 0.05% Triton X-100	90s/2mins	Separated but results in partially dechorionated/collapsed embryos
Bleach + Triton X-100	1-2hrs	2.5% active chlorine + 0.05% Triton X-100	90s/2mins	Separated but results in partially dechorionated/collapsed embryos
Bleach + Triton X-100	0-1hrs	1.25% active chlorine + 0.05% Triton X-100	2 mins	Separated and fully dechorionated but results in collapsed embryos
Bleach + Triton X-100	1-2hrs	1.25% active chlorine+ 0.05% Triton X-100	2 mins	Separated and fully dechorionated

Table 3.1: Embryo separation and dechorionation treatments

Treatments were evaluated for their ability to separate embryos from their clusters and either soften or remove the chorion to allow injection. After the treatment, embryos were transferred from a nylon mesh filter to glass slides for injection which also allowed assessment of their ability to withstand the necessary handling.

1). Exposure to NaOH or benzalkonium chloride solutions, as suggested in the literature for other lepidopterans[238] , at various concentrations had little effect on chorionic hardness. Protocols for dechorionation of *Drosophila* embryos using 2.5-5% sodium hypochlorite (bleach) solutions for 1.5-2 mins were able to dechorionate and separate embryos but resulted in embryos that were damaged on sides fully exposed to the bleach solution but that remained attached to their neighbours or still partially covered by their chorions. Longer incubations (>5mins) fully separated the embryos but nothing hatched. A weaker solution of 1.25% hypochlorite for 2 mins was therefore trialled, very similar to that used by Cosi et al. and Abidalla & Roversi[202,203], which resulted in embryos that were both fully separated and the majority completely dechorionated. I added 0.05% Triton X-100 (commonly used in *Drosophila* embryo wash) to all the bleach solutions, with the primary purpose of preventing embryos floating in the surface film or sticking to plastic surfaces. Cosi et al. reported that addition of another non-ionic surfactant, Tween 80, had a protective effect against mortality induced by bleach treatments, however I did not investigate the protective effect of low concentrations of Triton X-100. During all treatments embryos were agitated vigorously by either shaking at 350rpm or manually with a Pasteur pipette.

After dechorionation embryos were filtered through a homemade embryo collection basket and rinsed by submerging once in distilled water and then allowing a gentle stream of distilled water to pass over the collection basket filter cloth until the runoff no longer discoloured a piece of blue roll. Embryos dechorionated at 0-1hr PO were often too fragile to handle in this fashion. To counter this, embryos were collected after a 1hr oviposition window and allowed to develop for a further hour at 30°C before dechorionation, which enabled them robust enough for manual manipulation.

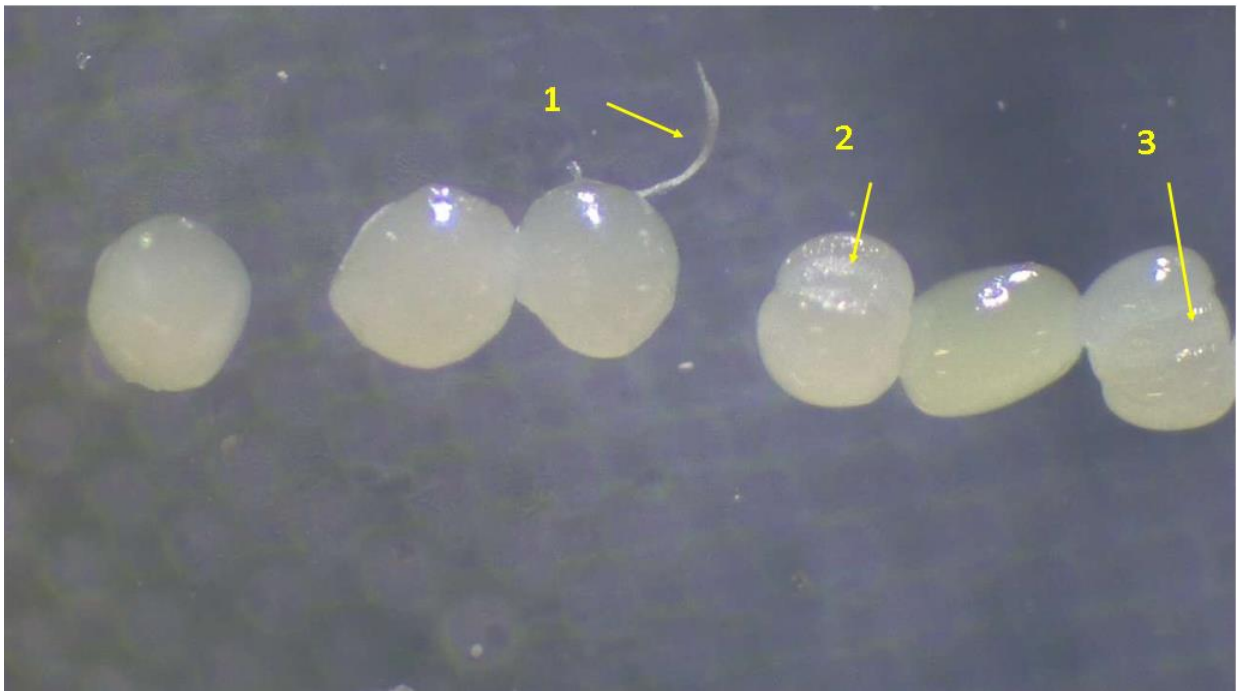

Figure 3.2: Partially dechorionated embryos

In this image we can see embryos that remain partially chorionated (yellow arrows). The remains of the chorion present as “wings” (1) or slightly dumbbell shaped (2,3) which can cause needle breakage during injection so should not be used.

3.3.2 Embryo Microinjection

Given that I was now able to reliably obtain large numbers of fully dechorionated, separated embryos, I set about optimising microinjection methodology. To inject *Galleria* embryos more easily, they were lined up in rows on either glass cover slips or glass slides using a sterilised paint brush cut down to a single hair. Any embryos that still possessed chorion remnants were not used. I found that fully dechorionated wet embryos placed on glass slides and allowed to dry slightly allowed sufficient adhesion such that physical manipulation by microinjection did not disturb them. However, to increase the consistency of the microinjection protocol, nail polish was used to 3 x 22x22mm glass cover slips in parallel to the 25x75mm glass slide. Embryos could then be individually aligned on the glass slides, resting against the cover slips to prevent them moving (Fig 3.4). To prevent further desiccation after aligning embryos, they were kept them in inverted petri dishes containing a 2% agar in water.

In *Bombyx mori*, it is thought that the germ band forms on the mid posterior-ventral surface of the embryo. Whilst it is unknown whether this is the same in *Galleria* embryos, and whilst they do not always have an obvious anterior or posterior pole when dechorionated where this could be deduced (embryos more oval than round), I aligned them such that the long axis ran parallel to the cover slip, and the more flattened surface (if present) parallel to the glass slide. The idea behind this was to increase the probability of injecting in the region where the germ cells would form compared to just injecting directly in the centre of the embryo.

For initial trials, injection solutions consisted solely of injection buffer, which was spun at 16,000g for 1 minute before loading to prevent needle clogging from

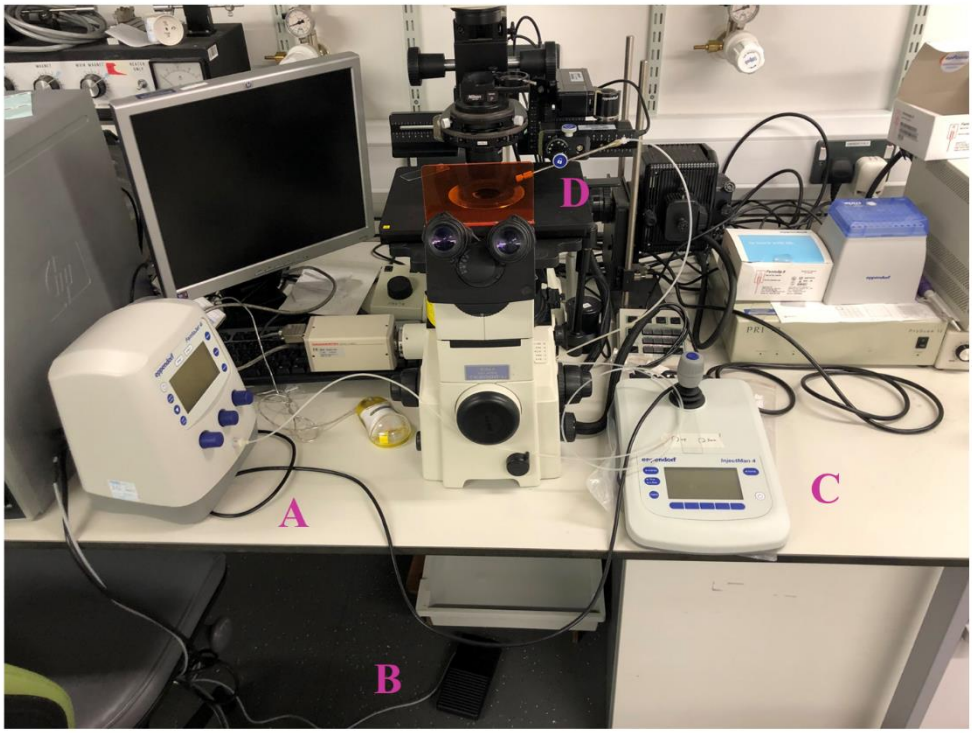

Figure 3.3: Microinjection Apparatus

The FemtoJet 4i pump (A) is connected to a foot operated injection pedal (B) and InjectMan 4 micromanipulator and injector (C). The InjectMan 4 controls the 3D position of the needle via a motorised needle holder attachment (D) directly mounted to the Nikon Eclipse TE-2000U inverted microscope.

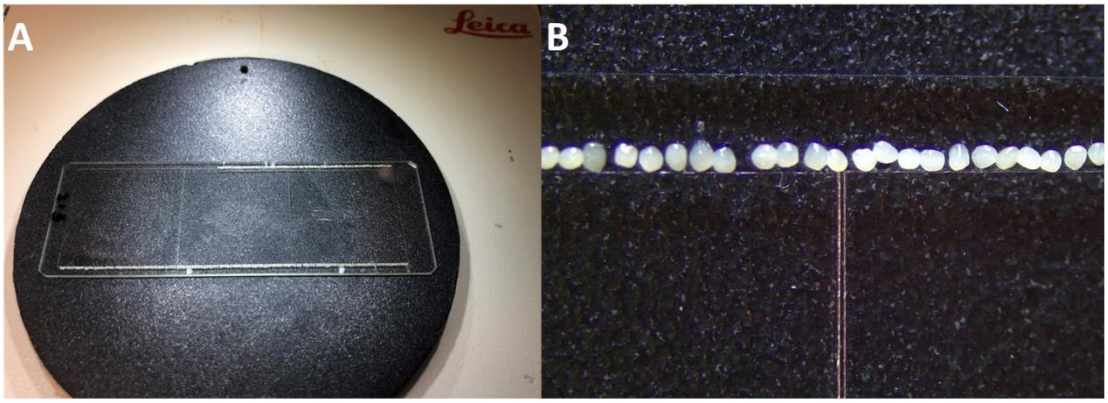

Figure 3.4: Embryo Alignment

3 x 22x22mm glass cover slips are glued to 75mm microscope slides using nail polish upon which roughly 300 embryos can be mounted (A). The small “shelf” formed by the glass slide on either side of the cover slips, can then be used to mount the embryos and provides a backstop to prevent embryo movement (B). The glass slide is also easier to handle than individual cover slips which helps to prevent accidental damage when moving the embryos during mounting, injection, rearing etc.

aggregated salt or dust particles, before loading into Eppendorf Femtotip microneedles which were attached to our microinjection system (Fig). I initially used Femtotip I needles, as I thought the stouter construction and shorter taper would protect against breakages from small bits of chorion still present. However, I eventually found this short taper to be a disadvantage – the narrow tip required high injection pressure and was prone to clogging on the viscous and sticky cytoplasm of moth embryos and attempting to clear it using pressure alone often proved unsatisfactory. I would therefore have to re-open the end of the tip against a glass slide, but the short taper and wider tip often damaged the embryo. I therefore switched to a related needle, Femtotip II. The longer taper made it easier to open the needle and gave a larger window of useable needle diameter. This allowed injection with less risk of clogging and little embryonic damage, but also allowed the use of lower pressures (350:75 hPa for injection:compensation pressures as a start) which reduced the disturbance caused to the inside of the embryo.

Embryos were injected by moving them onto the needle rather than by moving the needle itself. Frequently it was not necessary to inject them per se, as the compensation pressure was enough to observe a droplet forming in the embryo as a “clearing” in the cytoplasm. Injection volume was estimated from the size of droplet, and I aimed at ~0.5nl per embryo or a droplet roughly 1/5 diameter of the embryo corresponding to ~0.8% of the embryo volume. The embryos appeared to withstand changes in volume reasonably well without bursting and could be observed to “plump up” very slightly when correctly injected. However high internal pressure sometimes meant that the cytoplasm and injection mix could burst out the opening caused by the needle. It was therefore sometimes necessary to desiccate the embryos slightly by leaving out uncovered at RT for 5 or so minutes to prevent this. I

did not attempt to seal the injection opening in the embryos, as has been done when injecting other fully chorionated Lepidopteran embryos, as I found that embryo leakage over the following days not to be an issue, provided the embryo was correctly desiccated.

3.3.3 Post-injection rearing

Dechorionated *Galleria* embryos are vulnerable to over-desiccation so after injection, the glass slides holding the embryos were transferred to an inverted petri dish, with 2% agarose in the well of dish to maintain humidity and a strip of parafilm across the inside of the lid. Embryos were then kept at 30°C for 3-4 days, to allow them to develop until they were almost ready to hatch, which was indicated by the presence of eye pigmentation in the developing embryo.

The timing of embryonic development of *Galleria* varies considerably. When kept at 30°C, most emerged as L1 larvae over a period of 3 days, from day 5 to day 8. Initially, I transferred the glass slides holding the embryos directly to diet shortly before the first embryos hatch, thus allowing all larvae to feed. However, the *Galleria* diet used contains glycerol and other ingredients that readily absorb atmospheric water and I found this to desiccate the embryos causing them to die prior to hatching. Conversely, attempts to counter the desiccation by maintaining diet at high humidity caused the diet itself to become very sticky, and low survival of the hatched early larvae. I therefore trialled an agar version of the larval diet (5% agar, 20% larval diet by weight) to see if the newly hatched L1 larvae would readily feed on this from day 5-8 post injection, after which they could be transferred to normal dry diet. Larvae did not appear to feed well on an agar version, preferring instead to burrow into it, causing it to liquify and the larvae to subsequently drown.

To overcome these difficulties whilst maintaining high humidity around the embryos and lower humidity in the diet, I therefore placed the inverted agar petri dishes containing the embryos directly onto normal larval diet in the PET rearing jars with their agar lids still on. The petri dishes had vented lids, and the piece of parafilm

opened these up a little wider. Upon hatching, the larvae were able to crawl across the parafilm on the lid and down to the diet successfully. Only a few larvae were found stranded in the petri dish at 8 days; these were then carefully transferred to the diet using a single hair of a paint brush. Care was taken when doing this as I found the larvae could be attracted to static electrically charged surfaces.

To calculate the hatch rates following these manipulations, petri dishes were removed at 12-14 days post injection and observed under a dissecting microscope. As hatched L1 larvae leave very little trace of themselves on the slide, I found it easier to count embryos/embryonic remnants left on the slide at this time (i.e. unhatched embryos). Mould was occasionally seen growing on the remaining unhatched embryos after 14 days when calculating hatch rates, even after sterilising injection slides and embryo manipulators before use. Where excessive mould growth on the embryos themselves made this impossible hatch rates were noted as not done.

The hatched, injected larvae in the rearing tubs were then left to develop undisturbed. If they needed to be screened for phenotype (see Chapter 4,5), I found that it was best to do this either once they reach L3/L4 (around 1-1.5cm) to avoid harming the smaller larvae or once pupated. Pupae could be cut out of their pupal casings gently using a pair of scissors, thus allowing them to be sexed on the basis of their posterior morphology, prior to mating. The entire microinjection process is summarised in Figure 3.5.

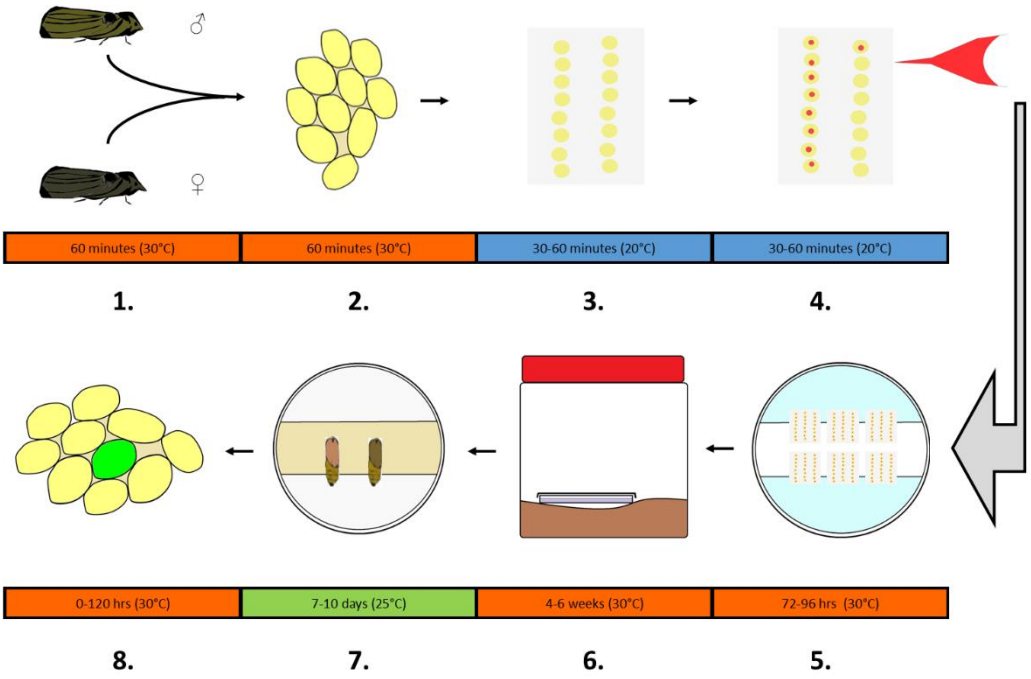

Figure 3.5 *Galleria* Microinjection Pipeline

Galleria injection and screening pipeline: **1.** Eggs are obtained from mixed sex adult *Galleria*. **2.** Embryos are left to develop to desired stage. **3.** Embryos are aligned on coverslip for microinjection. **4.** A small amount of microinjection mix is injected into the desired part of the embryo. **5.** Injected embryos are allowed to develop at high humidity. **6.** The embryos are moved to a jar containing larval diet, and the larvae are left to develop undisturbed until they reach late larval stage. **7.** Late larvae can be screened for phenotype or at the onset of pupation, pupae are removed from the sealed container and removed from their casings to allow sexual identification. Individual crosses between virgin injected and wild type adults can then be performed in petri dishes. **8.** Embryos collected from these crosses for screening

3.3.4 Effects of dechorionation and microinjection on survival

Early trials from the results described above had demonstrated that embryos could be dechorionated without resulting in excessive mortality. To quantify the effects that my protocol for dechorionation, handling and subsequent rearing procedures had on embryonic viability I subjected batches of 3-5 hour old embryos to either control or dechorionation treatments. In addition to this I also investigated how hatch rate success was impacted by injecting dechorionated embryos with moderate amounts of injection buffer.

The number of embryos used in each repeat and the number of repeats for each treatment differed. Batches of between 26-88 (N=732, 15 repeats) were used for untreated embryos, 80-400 (N=2481, 9 repeats) for the dechorionated embryos and 215-290 (N=1076, 4 repeats) for dechorionated and injected. Dechorionated and dechorionated + injected embryos were plated identically but untreated embryos were placed directly onto parafilm to prevent them moving during transfer. All embryos came from the same parent colony and had the same post treatment rearing.

Data from repeats was pooled and analysed for differences between observed and expected hatch rates using a χ^2 (Chi-squared) test, with pairwise Z tests with Bonferroni corrections used to determine significances in hatch rate differences between individual treatments. This showed that there were significant differences in hatch rates between both untreated embryos (87%) and dechorionated (77%) ones ($P < 0.05$) and untreated and injected (69%) ($P < 0.05$), as well as between those dechorionated and those which were injected ($P < 0.05$) as summarised in Figure 3.6. Although not quantified, the hatch rates recorded appeared to visually correlate

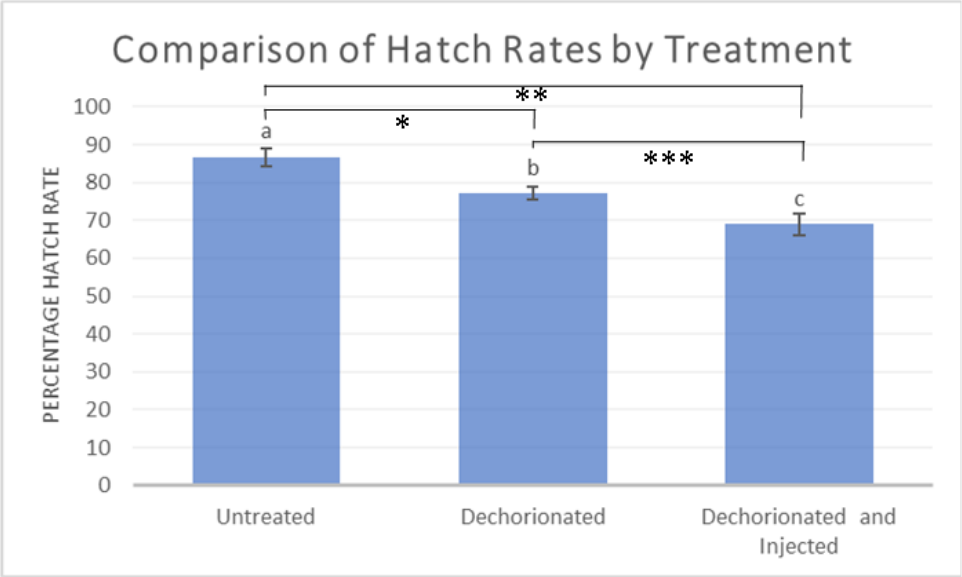

Figure 3.6 Effect of dechorionation and microinjection on embryo hatch rates

Legend: Hatch rates of untreated wild type embryos, and those that have been dechorionated or dechorionated and then injected. Different letters above bars indicate significant difference between treatments ($P < 0.05$, Pairwise Z-tests with corrections). All embryos were subjected to the same post treatment rearing. Error bars represent 95% confidence intervals. Asterisks indicate significance between treatments (P = <0.05)

with the number of larvae seen developing. Embryos from all treatments developed normally post hatching and were able to produce fertile offspring, with no observable difference in larval development between treatments.

3.3.5 Pre-blastodermal development and timings in *Galleria*

Ultimately, the optimised protocol for microinjection of embryos, recovery of larvae and rearing to adulthood was developed in order to be able to successfully inject mutagenic substances, such as PiggyBac transformation vectors. Such genetic transformation relies on incorporation of the DNA construct into the genome of germline stem cells. It was therefore crucial to understand the timing of early development of *Galleria* embryos. I therefore collected embryos and allowed them to develop to fixed time points, before dechorionating them and quickly fixing with a 1:1 heptane and methanol solution. Embryos were then rehydrated and stained with Hoechst 33258 to allow visualisation of nuclei under UV before imaging.

In embryos aged between 1.25 – 2.75hrs, sperm nuclei could be observed still in transit towards the ova nucleus and what appeared to be polar bodies migrating towards the boundary of the embryo. A small proportion of embryos in this time window could also be observed to have undergone their first mitotic division, with some beginning their second. From this I conclude that pro-nuclear migration and the first mitotic division occurs during this time (Fig 3.7).

In the next 3.5 hours, until 6.25hrs post oviposition, the number of nuclei increased rapidly as they migrated towards the periphery of the embryo (Fig 3.8). The nuclei were observed to maintain synchronous divisions throughout this period, indicating that they still share a common cytoplasm. This synchronicity was lost however by

8hrs post oviposition, indicating that cellularisation occurs between 6.25-8hrs. As embryos developed further, a difference in nuclei spacing became noticeable by 14hrs with the future embryonic tissue being more densely nucleated. I attempted to confirm cellularisation in 6-8 hr embryos by staining fixed embryos with an antibody that recognises the *Drosophila melanogaster* Septin-like protein, Pnut. However, despite a 68% protein sequence identity between *Drosophila* and *Galleria* for Pnut, only nonspecific cortical staining was observed.

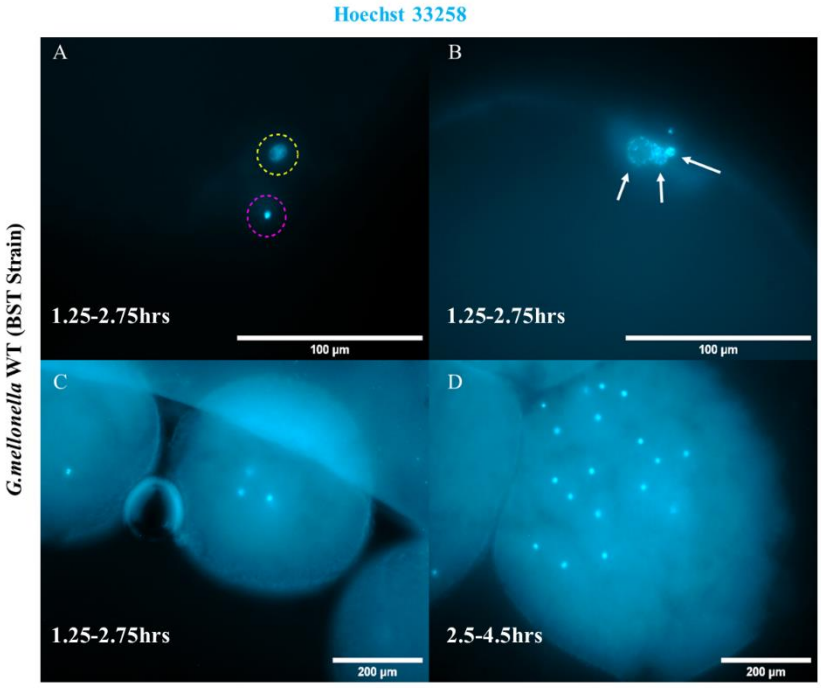

Figure 3.7 Zygote formation and initial mitotic divisions in *Galleria*

Galleria embryos fixed during set points in development and stained with Hoechst 33258 DNA dye. In the first 1.25-2.75hrs (Images A-C) post oviposition (PO) small nuclei, resembling sperm pronuclei (Image A – purple circle), can be seen moving towards the ovum nuclei (yellow circle) and nuclei that seem to resemble polar bodies gather towards the periphery of the embryo (Image B – white arrows). This timeframe appears to cover up to the second mitotic division (Image C). From 2.5-4.5hrs PO, energids migrate towards the periphery with an apparent bias for one pole (Image D).

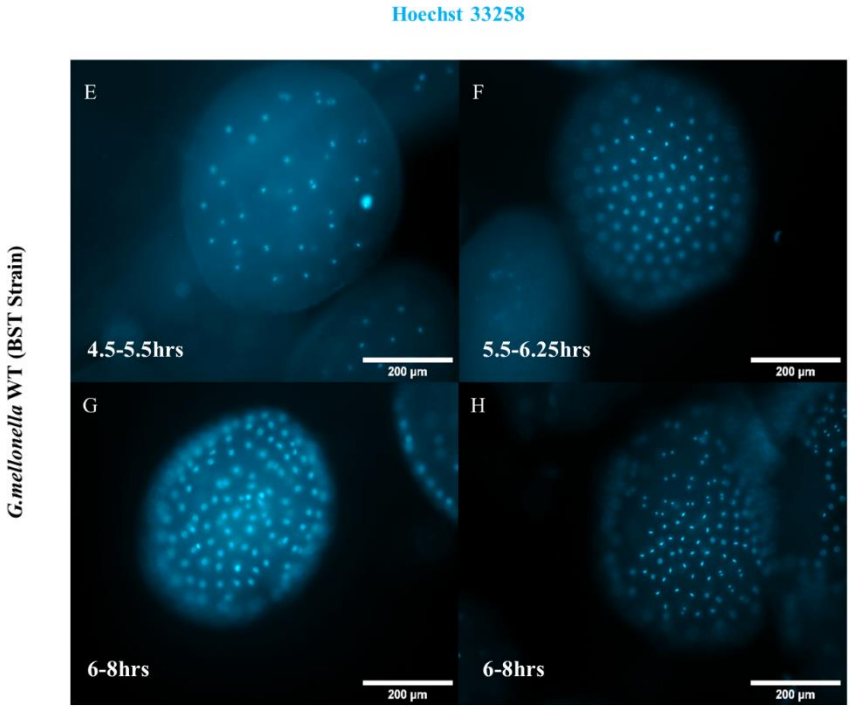

Figure 3.8 Early divisions of pre-blastodermal *Galleria* embryos

By 4.5-5.5hrs PO the first energids have just reached the periphery (Image E). Nuclei are dividing synchronously at this point as they share a common cytoplasm. All energids have reached the periphery by 6.25hrs and are still dividing together (Image F), although synchronicity begins to be lost from 6-8hrs (Images G & H) potentially indicating the onset of cellularisation.

3.4 Discussion

In this chapter I have described a method that I have developed and optimised that allows researchers to dechorionate, inject and rear early *Galleria* embryos to adulthood in the laboratory. The protocol presented is simple and, due to the high fecundity of this moth, a single researcher can prepare and inject from 600-800 embryos at a time in a 4-5hr window.

The techniques for rearing *Galleria* set out in both Chapter 2 and this Chapter are optimised to allow researchers to produce large numbers of developmentally matched embryos and to be require minimal maintenance and space. By putting down more embryos per week the rearing schedule will also produce large numbers of developmentally matched larval stages, allowing researchers to also have a regular supply of size matched larvae. The choice of diet has also been shown to produce immunologically robust larvae but comes with a downside in that the ingredients are more expensive. Dry goods can be bought in bulk relatively cheaply and can be stored for several years, but large amounts of honey are used regularly and remains expensive. A possible replacement would be sucrose dissolved in water, but this may have effects on larval immunity.

The experiments conducted to investigate suitable methods for dechorionation validate earlier findings by Cosi, Roversi an Abidalla[202,203] that a low percentage bleach solution for chorion removal in older (24hr) *Galleria* embros. Whilst I did not specifically investigate the potential protective effects that the addition of low concentration of non-ionic surfactant, such as Triton, added to dechorionation solution has on embryonic survival, I did observe that the age of the embryo at time of treatment has an effect. 0-1hr embryos were unsuitable for dechorionation with

1.25% active chlorine solutions of bleach since this rendered them so fragile that they collapsed under their own weight or at the slightest touch. This may be important for future transgenic techniques where time of injection can affect the efficiency of transformation.

Concomitant with the research described in the latter part of this Chapter, Abidalla[3] published a description of the morphogenesis and development of *Galleria* embryos from fertilisation to hatching. The staining of fixed embryos I have undertaken confirms that author's timings of early development with nuclei reaching the embryonic cortex at around 6 hours post-oviposition. It also confirms that *Galleria* follows a *Bombyx*-like development pathway up to cellularisation.

Most importantly this microinjection pipeline is the first to be described for this organism. It allows individuals to deliver microinjection cargo into large numbers of embryos in such a way that results in low embryonic mortality. As such it represents a large and necessary step towards the ability to create transgenic strains of this species, which will be discussed in chapter 4.

Chapter 4

Genetic Engineering *Galleria*

4.1 Introduction

The development of molecular and genetic tools that allow the creation of transgenic *Galleria* is one of the overarching goals for this project. Techniques mentioned in the previous chapter now allowed the reliable introduction of substances such as labelled proteins, antibodies or nucleic acids of our choice into *Galleria* at the embryonic stage and without inducing overly deleterious rates of mortality. This provided a very good basis upon which to start attempting to generate transgenic strains of *Galleria*.

Ideally methodologies for both inserting new genetic material and removing or impairing the function of endogenous genes would be applied to this organism, as gain and loss of function studies have been crucial to understanding molecular and systemic pathways in other organisms. With this in mind, two techniques were focussed upon in *Galleria* – both for their previous application in Lepidopteran systems[109,147] and because their broad range of use in a wide variety of systems has meant that the protocols and resources were available.

PiggyBac transgenesis was chosen as the method for introducing new genetic material. This transposase-based technology, of lepidopteran origin[101], has been shown to integrate very large genetic cargos in multiple organisms and a wide variety of lepidopteran specific donor and helper plasmids already exist. It has advantages over other transposase systems in that it integrates seamlessly into the genome, and hyperactive versions of the transposase have been engineered to improve efficiency. Unlike phiC31 integrase-based techniques however, its insertion

site is semi-random with a preference for open chromatin and therefore has the potential to disrupt endogenous genes or integrate at a genomic site that is transcriptionally silent or repressed.

For genomic knockouts, CRISPR/Cas based technologies seemed the obvious solution. Advances in CRISPR technologies over the last 10 years has meant that this tool is able to provide the same sort of outcomes as RNAi and ZFN/TALEN based knockdown and knockout techniques. The advantage of CRISPR though is in its greater adaptability and reduced labour cost for the researcher due to both the nature of the protocol and the increased availability of commercial CRISPR products.

In this chapter, I describe how these tools were applied to *Galleria*, the adaptations necessary and success achieved in this model.

4.2 PiggyBac Mediated Transgenesis

4.2.1 PiggyBac donor reporter plasmid activity in *Galleria* embryo

At the start of the project, the Wakefield lab was in possession of 5 PiggyBac reporter plasmids, that had been obtained through the aforementioned Innovate UK grant. These are summarised in Table 2.1, but consisted of 2 vectors with EF-1α driven expression of GFP or RFP (PB531A and PB513B), and 3 vectors with insect promoter driven GFP (p*Bm*hsp90:GFP/3xP3:DsRed, pPIGA3GFP and pBac[Pub-nls-EGFP]).

Before commencing any large scale attempts to generate transgenics using these plasmids, I first sought to determine the activity of their respective promoters during *Galleria* embryogenesis by injecting the plasmids on their own at 900ng/μl into 20 to 30 3-4hr embryos per construct and then monitoring their fluorescence over the course of 96hrs. No expression was seen for either PB531A or PB513B, possibly inferring that the human EF-1α promoter is unable to drive reporter activity in the early *Galleria* embryo. Mosaic expression was seen however, in embryos injected with any of the three reporters containing insect promoters (Fig. 2.1). The GFP expression pattern consisted of puncta of fluorescence within the embryo and it was difficult to identify whether there was any specific localisation to any tissue.

The plasmid p*Bm*hsp90:GFP/3xP3:DsRed was chosen as the donor reporter plasmid to use for further experiments based on the reporting by Tsubota et al[204] that the *Bombyx* hsp90 promoter had higher activity in cell lines compared to the A3 cytoplasmic actin promoter, and was active in all tissues compared to the A3 promoter had been reported to not be active in embryonic tissue[77,205]. Unlike other heat shock protein promoters, hsp90 is constitutively active in Lepidoptera. Whilst

Drosophila poly-ubuiqitin promoter has been shown to drive strong constitutive expression in *Drosophila*, it has not been widely used or reported in lepidoptera and so I considered the *Bombyx* hsp90 promoter to be a safer choice. The vector p*Bm*hsp90:GFP/3xP3:DsRed has another advantage in that it contains a second reporter – 3xP3 eyeless promoter driving DsRed – which has been shown to express in a wide variety of organism[121,204,207,208] and could be useful in the case of high levels of autofluorescence in the GFP channel or low activity of the hsp90 promoter.

Within the literature, the figures for concentration of donor PiggyBac plasmids for injection varies from as little as 200ng/µl up to around 800ng/µl[77,209]. To see if the plasmid concentration had any effect on transient embryonic expression in *Galleria*, I injected early embryos p*Bm*hsp90:GFP/3xP3:DsRed at a range of concentrations (150, 250, 400 and 800ng/µl) and monitored them for 96hrs. GFP expression, in the form of puncta of fluorescence within the embryos, was observed for all concentrations with very little difference observed in either the intensity or the proportion of fluorescent embryos between treatments.

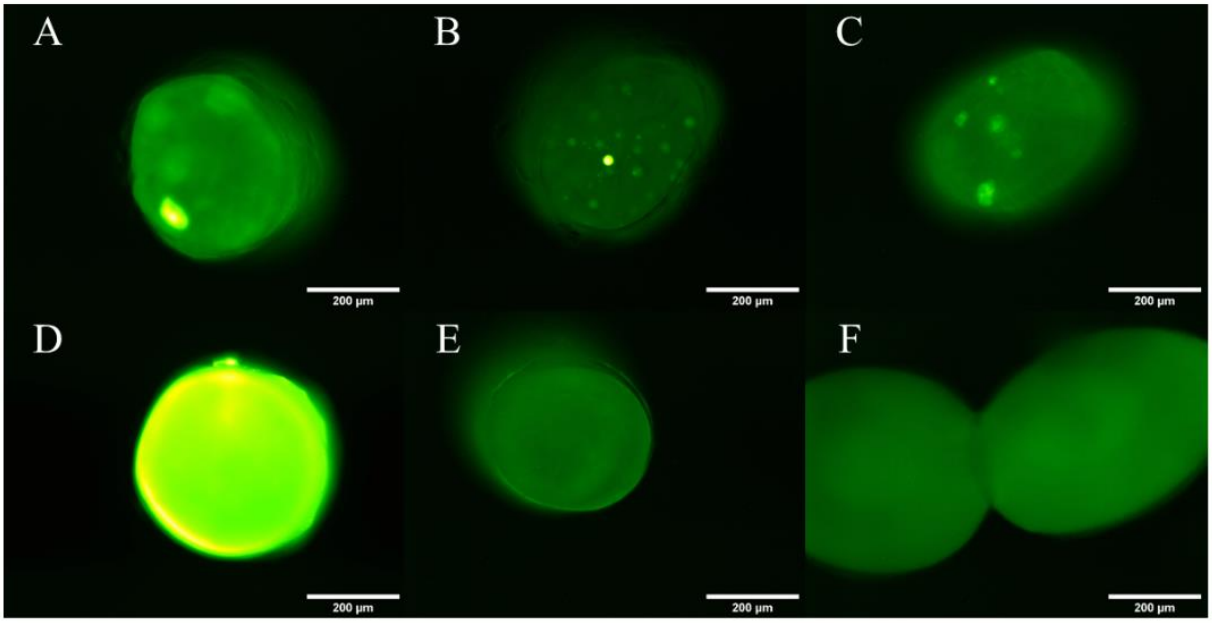

Figure 4.1: Injection of PiggyBac donor plasmids into *Galleria* embryos

False coloured images of embryos that were injected at 3-4hrs post oviposition and imaged at 48-72hrs old. Images A-C are representative embryos injected with one of 3 different PiggyBac constructs that contain insect promoter driven GFP reporter sequences. In addition to this are positive and negative controls (Images D and E respectively) and uninjected embryos (Image F). Mosaic GFP expression can be observed in embryos injected with p*Bm*hsp90:GFP/3xP3:DsRed, pPIGA3GFP and pBac(Pub-nls-EGFP) (Images A, B and C respectively). Injection of GFP protein fluoresced brightly up to 72hrs post injection (Image D) but injection of standard injection buffer induced no autofluorescence (Image E).

4.2.2 Attempted transgenesis using the helper plasmid pHA3PIG

p*Bm*hsp90:GFP/3xP3:DsRed, and in particular the *Bombyx* hsp90 promoter, was shown to have some activity *in vivo* in the *Galleria* in the early embryo and despite not being observed it also seemed a reasonable assumption that the 3xP3 eyeless promoter would be active too given its use in other insects.

I therefore set about attempting to generate a transgenic strain of *Galleria* using this as the PiggyBac donor and pHA3PIG as the helper. These plasmids were ethanol precipitated after propagation in *E.coli* and resuspended in a phosphate/KCl injection buffer. A concentration of 200:200ng/µl (400ng/µl total) of donor:helper for the injection mix was chosen initially. Whilst higher concentrations of donor and helper might increase efficiency, I reasoned that using lower concentrations should reduce mortality and previous methodologies using these concentrations had consistently achieved transgenesis in *Bombyx* using the same helper.

Over 4000 embryos were injected using the methodology described in the previous chapter, of which 28.2% hatched and 13.7% developed until pupation (Table 4.1). A subset of these (~500) were screened for fluorescence at the G0 larval stage, of which only one showed a patch of fluorescence that was localised to the side of one of its segments (Fig. 4.2). G0 pupae were carefully removed from their pupal cases and sexed. The large number of pupae to be mated, meant it was not only extremely time consuming for the author but also that frequently there were not enough wild type pupae to mate the G0 injectees. The G0 pupae were therefore mated to a mixture of wild type and siblings in petri dishes. The mating dishes never contained wild types of both sex in a single dish, and numbers per petri dish were kept between 2-4 moths.

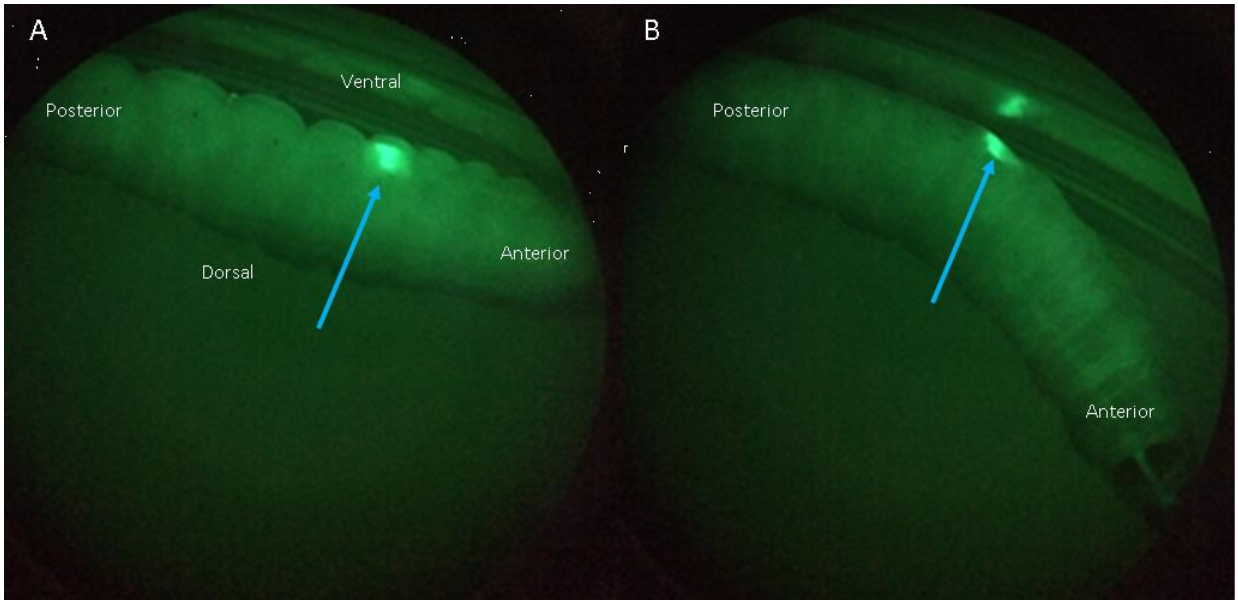

Figure 4.2 Somatic mosaic G0 larvae injected with pHA3PIG helper and p*Bm*hsp90:GFP/3xP3:DsRed donor plasmid

G0 last instar larvae, screened at roughly ~6 weeks after injection with a 1:1 mixture of pBachsp90GFP-3xP3DsRed:pHA3PIG (total conc. 400µg/ml), displaying somatic fluorescence (blue arrows). Fluorescence is localised to the ventro-lateral aspect of a body segment roughly halfway down the anterior/posterior axis (Image A), and can be determined to be internal fluorescence and not an artefact (Image B). This indicates the *Bombyx mori* hsp90 promoter is able to induce robust expression in somatic larval tissue.

G1 embryos were collected from these crosses and screened for fluorescence at 72-96hrs post oviposition, however no embryonic fluorescence in either the GFP or DsRed channels was observed in any egg clusters. They were then placed into 15ml falcon tubes containing larval diet, and the perforated falcon tube lid used to secure a piece of 50μm steel mesh, where they were allowed to develop until hatching. In several crosses the embryos died during late embryogenesis failed to hatch or the L1 larvae died shortly hatching, however it wasn't clear whether this was due to the injection process or other environmental factors such as overly dry diet.

Whilst autofluorescence from the larval diet made it harder to screen the larvae when smaller and in their tubes, no GFP or RFP fluorescent individuals were seen in the crosses at L1 or any other later larval stages. From this it was concluded that the injections with these plasmid mixes and concentrations were not successful.

4.2.3 A hyperactive PiggyBac donor is able to generate transgenic *Galleria*

When the first attempt to create a transgenic strain of *Galleria* did not succeed using the earlier injection parameters, it appeared that there could be an issue with either the injection protocol or the plasmids themselves. It seemed likely that the donor reporter plasmid could persist and integrate the *Galleria* genome, but that the rate at which this was happening was very low.

A dual approach was taken to try and improve efficiency before the next experiment. To start with, a second helper plasmid was created using the mammalian codon optimised hyperactive transposase which had previously been shown by Eckermann et al[108] to be more effective than the wild type - p*Bm*hsp90hyPBase. This used the *Bombyx* hsp90 3kb upstream sequence from p*Bm*hsp90:GFP/3xP3:DsRed to drive the expression of the mammalian codon optimised hyperactive PiggyBac transposase CDS from PB210PA-1 (Table 2.1), with the *T. ni* PiggyBac transposase UTR from pHA3PIG. In addition, the concentration of donor plasmid was increased from 200 to 400ng/µl.

For the next round of injections, a much smaller number of embryos were used. 2 x 400 embryos were injected with a 400:200ng/µl ratio of donor:helper plasmid, both using p*Bm*hsp90:GFP/3xP3:DsRed for the donor, but with one set of 400 using the original pHA3PIG helper and the other p*Bm*hsp90hyPBase. The hatch rates were not recorded for these injections, but pupation rates were 12.7% and 20.8% respectively (Table 4.1), with similar survival between this set and the previous pHA3PIG injections and slightly higher survival for p*Bm*hsp90hyPBase. Similar to the previous batch of injections, a proportion of G0 larvae (~30 individuals for each batch) were examined for fluorescence from both groups. No fluorescent larvae were

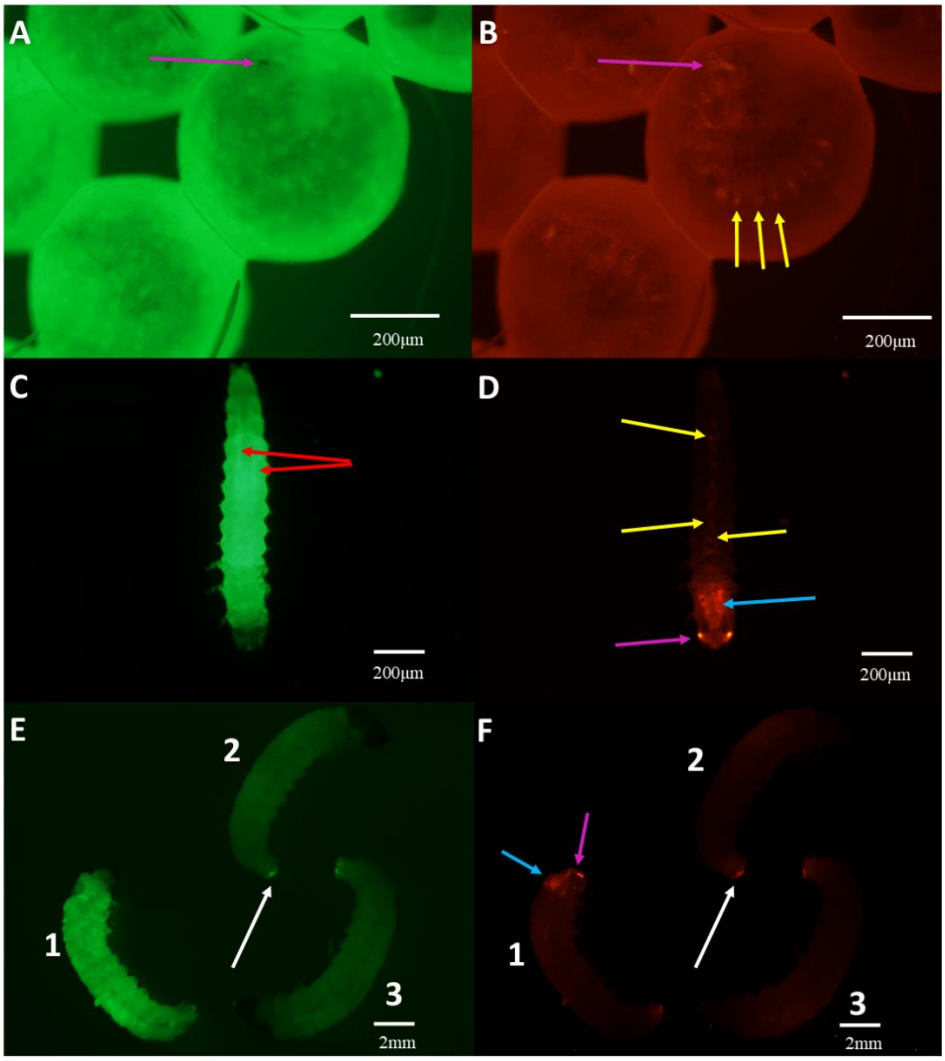

Figure 4.3 G1 *Bm*hsp90:GFP/3xP3:DsRed2 Embryos and Larvae

Galleria expressing GFP under the control of the *Bombyx* hsp90 promoter and DsRed2 under the 3xP3 promoter, appear not to express GFP in embryonic germ tissue, but can be observed in yolk and vitellophages (A). At day 4 DsRed2 can be seen in embryos, and in pharate larvae expression seen in the eyes (purple arrow) and segmental ganglia (B, yellow arrows). In L1 larvae (C-F), GFP is brightest in the malpighian tubules (C, red arrows) and GFP expression brightest in eyes and brain (D, purple and blue arrows respectively). In both GFP and RFP channels, transgenic larvae (1) are noticeably different from wild type larvae (2 & 3) but it is worth noting that larval frass is autofluorescent.

observed for the pHA3PIG larvae, but 6 individuals with mosaic GFP expression observed in the p*Bm*hsp90hyPBase larvae. In addition, a single p*Bm*hsp90hyPBase pupae was observed with mosaic RFP fluorescence in one eye, indicating that the 3xP3 promoter is active in eye tissue in *Galleria*.

To try to maximise the chances of G0 reproductive success survival and to minimise the impact of any G0 infertility, no sibling matings were performed in this set of injections. Instead all G0 pupae were crossed to two WT pupae of the opposite sex. G1 Embryos were once more screened at 72-96hrs post oviposition for fluorescence in GFP and RFP channels, with no fluorescence seen in embryos from any of the crosses from either injection treatment. Embryos were then placed onto freshly made diet in polystyrene fly vials (flystuff) and sealed with a cotton bung before incubating at 30C until hatching.

G1 larvae were screened in their vials when at L1 stage and again but separated from the diet when they reached L3/L4 (~1cm long). A single cross was found to contain 6 transgenic larvae, which fluoresced in both GFP and RFP channels. GFP expression was visible across the whole larva but appeared brightest in the Malpighian tubules, however was difficult to distinguish from autofluorescence coming from the diet and the larval frass. Eye/neural specific RFP expression was clearly visible in both pharate larvae and L1 individuals and was a more useful screening channel against the diet.

Transgenic G1 larvae were separated out from wild type G1s when large enough to safely handle and reared individually until pupation. They were then outcrossed for two generations before sibling mating. A homozygote line was created by selecting the brightest larvae from this fraternal cross for mating, which was then maintained

without any outcrossing. Analysis by iPCR showed that the transgene cassette has TTAA sequences at either end, implying it was inserted seamlessly within an endogenous TTAA sequence. It appears to be located intergenically,between LOC113512719 and LOC113515287 both of which are putative inorganic phosphate co-transporters (Supplemental material 3). BlastP searches on the translated features reveal homologous proteins (>50% accession ID, E=0) in *Bombyx mori* have been mapped to chromosome 8. Larvae have been maintained as homozygotes for 15 generations since indicating that the homozygote is viable.

4.2.4 PiggyBac transgenesis mediated expression of fusion proteins

With the first strain of transgenic *Galleria* now created, I decided that it would be beneficial to investigate the use of the PiggyBac transposon system for generating transgenic strains expressing fusion proteins that allow tracking of cellular and subcellular processes. Technologies such as this would be beneficial for studying the cellular origins of immune response and pathogen binding mechanisms in future infection studies, as well as for developmental or cell biology experiments.

Strains expressing fusion products with easily visible and distinct localisations would be ideal for a proof of concept study and would demonstrate both the feasibility and potential use of this technology. Given the background of the Wakefield lab, I chose to generate two strains with cellular phenotypes involved in mitosis. A large part of the Wakefield group's work looks at microtubule spindle formation and chromosome segregation, so a strain that visibly labelled microtubules and chromatin would allow us to look at these structures in *Galleria*. Within the lab and the wider field of cell division, *Drosophila* strains expressing labelled α tubulin and histone proteins are

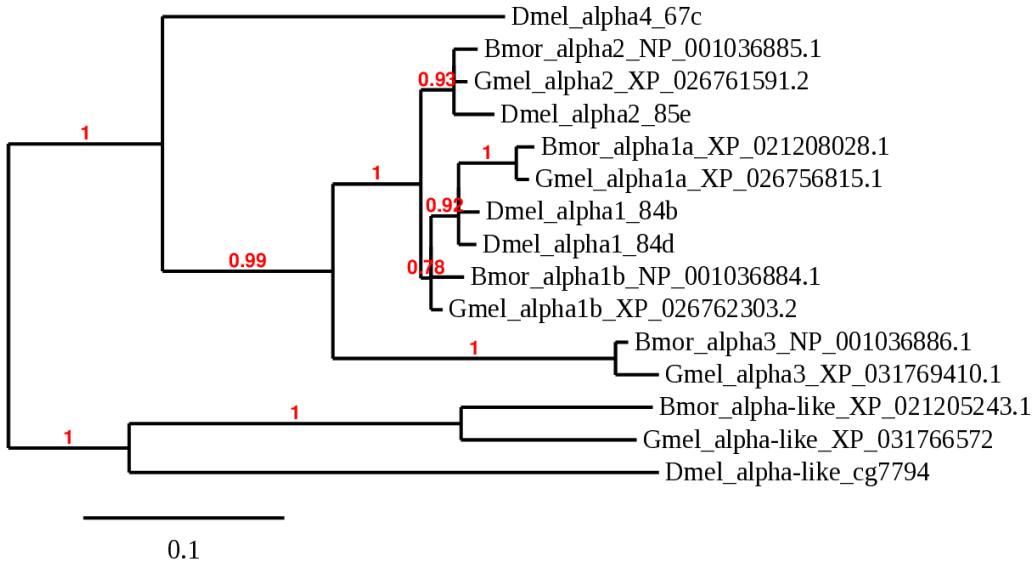

Figure 4.4 Phylogram of *Galleria*, *Bombyx* and *Drosophila* α-Tubulins

Phylogenetic hypothesis of α-tubulins from these 3 species inferred by Mr Bayes 3.2. Values in red indicate posterior probablilty support for each clade. The α-tubulins appear to fall into distinct classes. The constitutively expressed α-tubulins in each organism fall within the α1 class, however the expression pattern for one of the *Galleria* and *Bombyx* α1s (1a) has not been documented. The α2 class is represented in all 3 organisms and is known to be expressed neuronally in *Bombyx* and *Drosophila*[210,211]. α3 is only found in *Bombyx* and *Galleria* and has been previously described as testes specific[211]. The ovary/maternally derived embryonic specific α4 class tubulin was not present in either species of moth, but has been widely studied and described in flies. A poorly described class of α-tubulin like genes was found in all 3 species, which have shown to be expressed in *Drosophila*, but whose function is unknown.

commonly used to visualise mitosis in real time and seemed sensible to copy this approach.

Drosophila possesses 4 α tubulin genes and α tubulin-like gene (CG7794). Of the α tubulins, two (84B and 84D) are from α family 1 and expressed in all life stages210, although 84B is slightly more abundant and found in a great number of tissues. 85E, of the α family 2 is expressed primarily from late embryogenesis onwards into adulthood but more highly expressed in the adult male than female. α tubulin 67C is a divergent α tubulin of the α tubulin family 4 and primarily expressed in the adult female germline cells and embryonic tissue242 with the properties of this specific tubulin necessary for normal development and thought to be involved in the rapid cycles of syncytial cell division that characterise early embryonic development[210]. In *Bombyx* mori, there are 3 reported 3 α tubulins (NP_001036884.1, NP_001036885.1 and NP_001036886.1) found from EST databases[211], falling into the the α tubulin 1, 2 and 3 family respectively. Blast searches of the *Bombyx* genome using *Drosophila* α tubulins revealed another documented but undescribed α tubulin 1 gene (XP_021208028.1) and an α tubulin-like gene (XP_021205243.1). Using these previously known and newly described tubulin species from *Bombyx* and *Drosophila*, blast searches revealed 4 α tubulin genes in *Galleria* and one α tubulin-like gene. It did not appear that full transcripts were for two of these proteins (XP_026761591.2 and XP_031769410.1) on the refseq database, so using the *Bombyx* mori homologues as a guide these protein sequences manually annotated against the Korean *Galleria* genome. These searches indicated, perhaps unsurprisingly, that the *Bombyx* and *Galleria* tubulin proteins were highly conserved with each possessing two α tubulin 1 genes and one α tubulin 2 and α tubulin 3 gene, as well as 1 α tubulin-like gene. In *Bombyx* the main ubiquitously expressed tubulin is

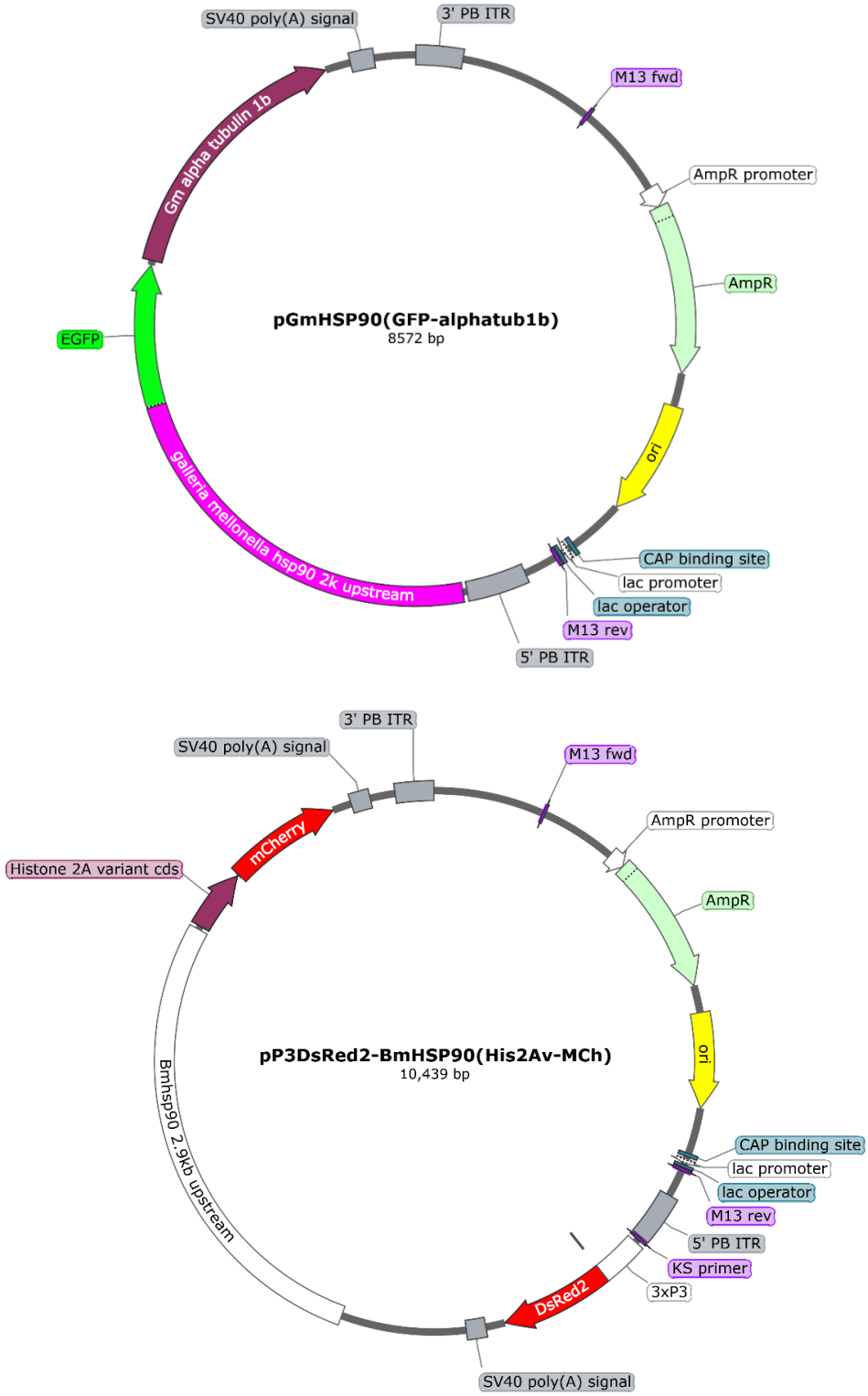

Figure 4.5 Plasmid maps for p*Gm*hsp90:(GFP-αtub1b) and *pBm*hsp90:(His2av-mCh)/3xP3:DsRed2

NP_001036884.1 (henceforth labelled Bmα1b) with the α2 and α3 family tubulins expressed in neural tissue and testes respectively. The expression pattern of the other two tubulins, α1a and α-like is unknown.

To ascertain which is the ubiquitously expressed α tubulin in *Galleria*, RNA extracted from embryonic and larval end segment tissue was screened via RT-PCR with 2 pairs of primers for each of the *Galleria* α tubulins. Only α tubulin 1b was found to be expressed to a detectable level through this screen in these tissues (Fig 4.6), so this was chosen as the gene to insert as a fluorescently labelled transgene.

In *Drosophila* the majority of histones are encoded by polycistronic genes, which can make generating expression constructs tricky, so instead the monocistronic histone 2av gene to avoid these issues. A BLAST search revealed that *Galleria* has a single hit for this *Drosophila* protein, indicating a likely orthologue. Manual inspection of the CDS in the Korean genome revealed that this was likely to be monocistronic and therefore suitable for this study.

Transgenic donor constructs were generated via Gibson assembly as before, with the α tubulin tagged on the N terminal and the Histone 2aV tagged on the C terminal as previously described by Grieder et al. and Kotova et al.[212,213]. The α tubulin 1b gene was amplified from extracted mRNA using primers (α1bCDS-F and α1bCDS-R), and inserted into AgeI/KpnI digested Gmhsp90:GFP backbone. Histone-mCherry + SV40 PolyA signal was synthesised by Geneart with homology arms corresponding to PmlI/AscI digested pBmhsp90(GFP)3xP3(DsRed). The geneart insert also contained the very 3’ end of the Bmhsp90 upstream region, but with the native Kozak sequence and an extra AsiSI site for easier cloning later down the line.

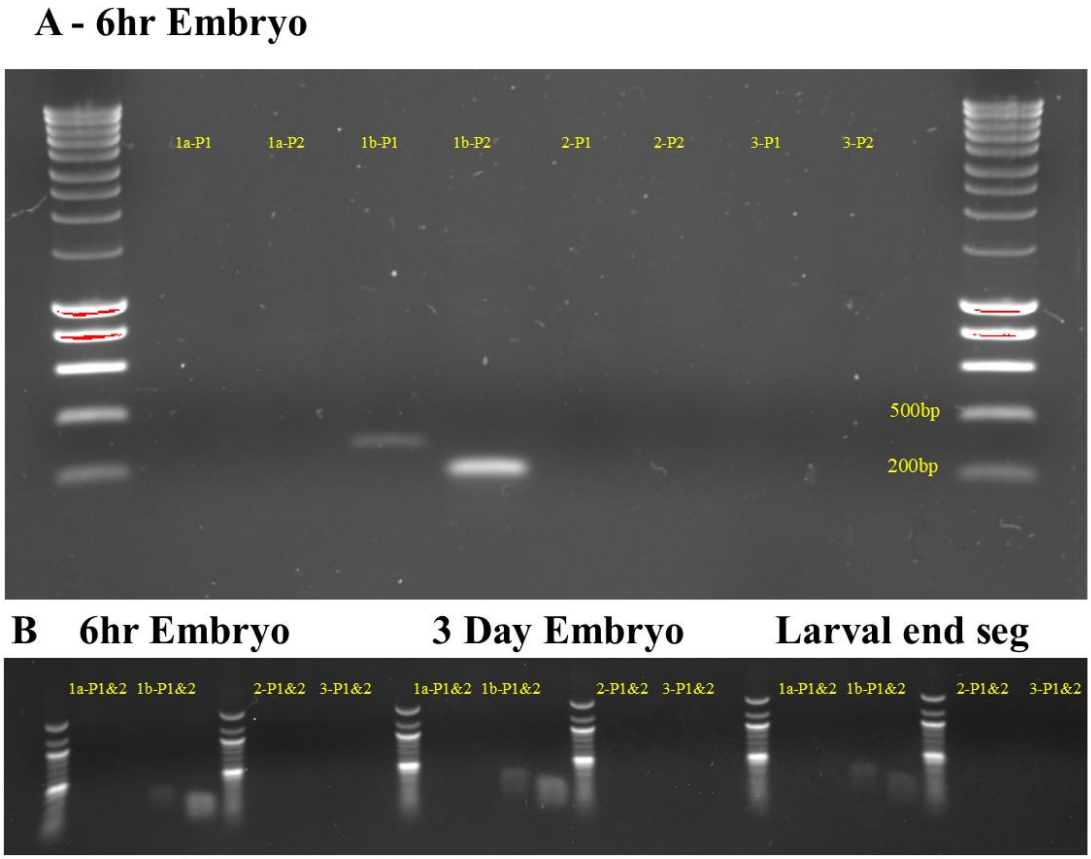

Figure 4.6 RT-PCR of α-tubulins 1a, 1b, 2 and 3 in embryo and larval tissue

Only α-tubulin 1b is detected in the 6hr old and newly cellularised embryo (A), however tubulins 1a, 2 and 3 are not detectable in 3 day embryo or in the end segement of a 4th instar larvae. P1 and P2 refer to Rt-PCR primer pairs 1 and 2 for each tubulin mRNA.

mCherry was chosen over DsRed for the histone label to avoid any interactions between the RFP moieties as DsRed is known to tetramerise. The endogenous tubulin and histone promoters were not used, partly because I had constructs already available with the hsp90 promoter already present, but mainly because I wanted the constructs to drive strong constitutive expression at all life stages and was unure as to how expression of these two genes varied during development.

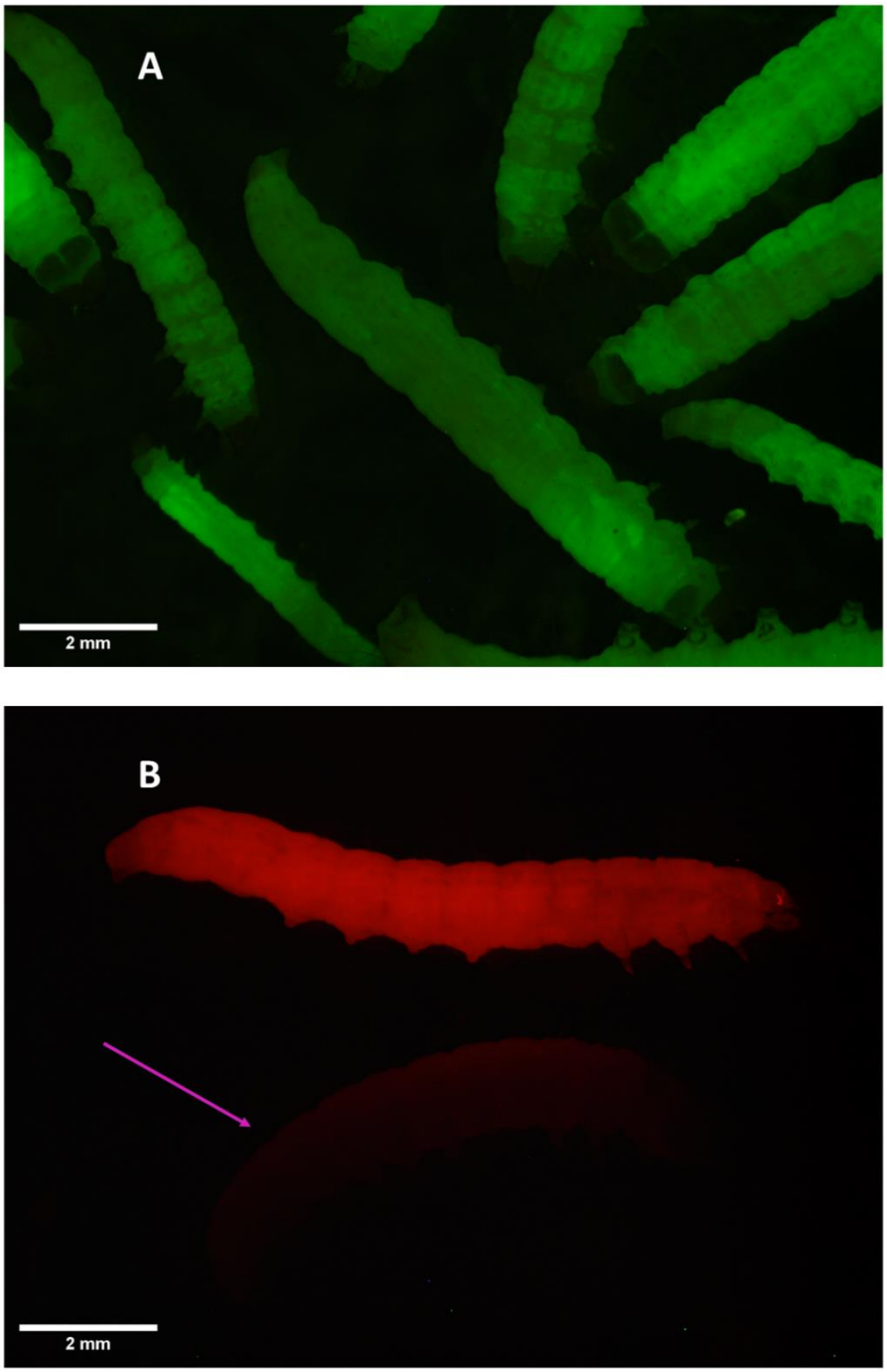

Figure 4.7: *Gm*hsp90:(GFP-αtub1b) and *Bm*hsp90:(His2av-mCh)/3xP3:DsRed2 larvae

*Gm*hsp90:(GFP-αtub1b) larvae (A) and *Bm*hsp90:(His2av-mCh)/3xP3:DsRed2 larva (B). Purple arrow in image B indicates the WT larva below the transgenic larva, showing little autofluorescence in this channel.

Transgenic individuals were then generated as described in the previous section, with the only divergence from that described protocol being the use of a slightly higher concentration of donor plasmid (500 vs 400 ng/ul). Hatch rates for both sets of injections were very similar at ~26% for both constructs which seems to correlate with what was observed for the initial injections with p*Bm*hsp90:GFP/3xP3:DsRed and pHA3PIG. Pupation rates for the two constructs however did differ markedly, with 19.5% and 6.0% for GFP- αTub1b and His2aV-mCherry respectively. One explanation for the lower pupation rate is that it is possible that the His2av-mCherry construct is slightly more toxic, but could also be due to other factors such as varying low moisture content in the larval diet which our lab has found can affect development.

G1 embryos were placed directly into larval jars in batches of 5 crosses per jar. Whilst this pooling of embryos would likely lead to a lower discovery rate of strains from individual crosses, the G1 larvae developed more consistently and allowed a larger number of larvae screened. In some cases in smaller tubes it was observed that overcrowding lead to L1 death, which meant only a small number of embryos could be added to the tube.

Due to the pooled G1 larvae it was conservatively estimated that any transgenic larvae from the same jar were from the same strain. Two different strains were found for the GFP- αTub1b line and 1 for the His2aV-mCherry line. Individuals from both the GFP- αTub1b lines showed variation in their fluorescence locations and intensities, however the His2aV-mCherry individuals had a consistent phenotype. The brightest of all the individuals with the most uniform fluorescence in these lines were outcrossed for two generations before sibling mating. iPCR was unsuccessful in locating transgene insertions within the genome for either strain.

Constructs	Injection concentration	Strain injected	No. Injected	No. Hatched	No. Pupated	Positive Broods
p*Bm*hsp90:GFP/3xP3:DsRed + pH*Bm*A3:PIG	200ng/ul 200ng/ul	WT	4692	1323	641	0
p*Bm*hsp90:GFP/3xP3:DsRed + pH*Bm*A3:PIG	400ng/ul 200ng/ul	WT	400	Not Done	51	0
p*Bm*hsp90:GFP/3xP3:DsRed + p*Bm*hsp90:hpPBase	400ng/ul 200ng/ul	WT	400	Not Done	83	1
p*Gm*hsp90:GFP-αtub1b + p*Bm*hsp90:hpPBase	500ng/ul 200ng/ul	WT	1450	372	283	2
p*Bm*hsp90:histone2av-MCh/3xP3:DsRed + p*Bm*hsp90:hpPBase	500ng/ul 200ng/ul	WT	1280	327	77	1
GFP-sgRNA + Cas9-MCh protein	2000ng/ul ~800ng/ul	*Bm*hsp90:GFP/ 3xP3:DsRed	400	122	28	14

Table 4.1 Injection Data for PiggyBac and CRISPR mutagenesis experiments

Summary table for all injection mix parameters and survival rates of microinjection experiments described in this chapter at different life stages in *Galleria*. Where hatch rates were not calculated they are noted as not done.

Although it is not a direct comparison due to the GFP-tubulin fusion and the shorter hsp90 upstream sequence for the GFP- αTub1b strain provided a chance to compare the expression profiles of *Galleria* hsp90 promoter and the *Bombyx* one used in the *Bm*hsp90:GFP/3xP3:DsRed line.

Overall, larvae of the *Bombyx* promoter appeared brighter with consistent expression along the length of the body and, at low magnification, dissected tissues could be seen to strongly express GFP in the malpighian tubules but weakly in the gut. Larvae expressing GFP under the control of the *Galleria* promoter were dimmer overall with expression focussed more towards the anterior pole in larvae, and most notably observed in the gut with the malpighian tubules only expressed GFP weakly. In some individuals of the GFP- αTub1b strain, the fluorescence in the visceral fat body reduces in intensity towards the posterior of the larvae however this varies between individuals and generations. This variance may be due to differential activation of the additional hsp90 promoter due to environmental factors, instability of the transgene within the genome or insertion locus specific regulatory effects. Both strains showed similar patterns of strong expression at the anterior end of the mid silk gland with little elsewhere in this organ, and no expression in tracheal tissue.

It wasn’t possible to ascertain whether neural tissue expressed GFP under the *Bombyx* hsp90 promoter, due to red channel fluorescence bleed through from the co-expressed DsRed under the control of the 3xP3 promoter, but GFP expression was not observed in the *Galleria* hsp90 strain. Unusually, neither promoter was expressed in embryonic germ tissue, despite this being the case in *Bombyx* and RT-PCR and EST databases showing that the endogenous *Galleria* hsp90 gene was transcribed in embryos. Instead *Bombyx* hsp90 promoter activity was observed

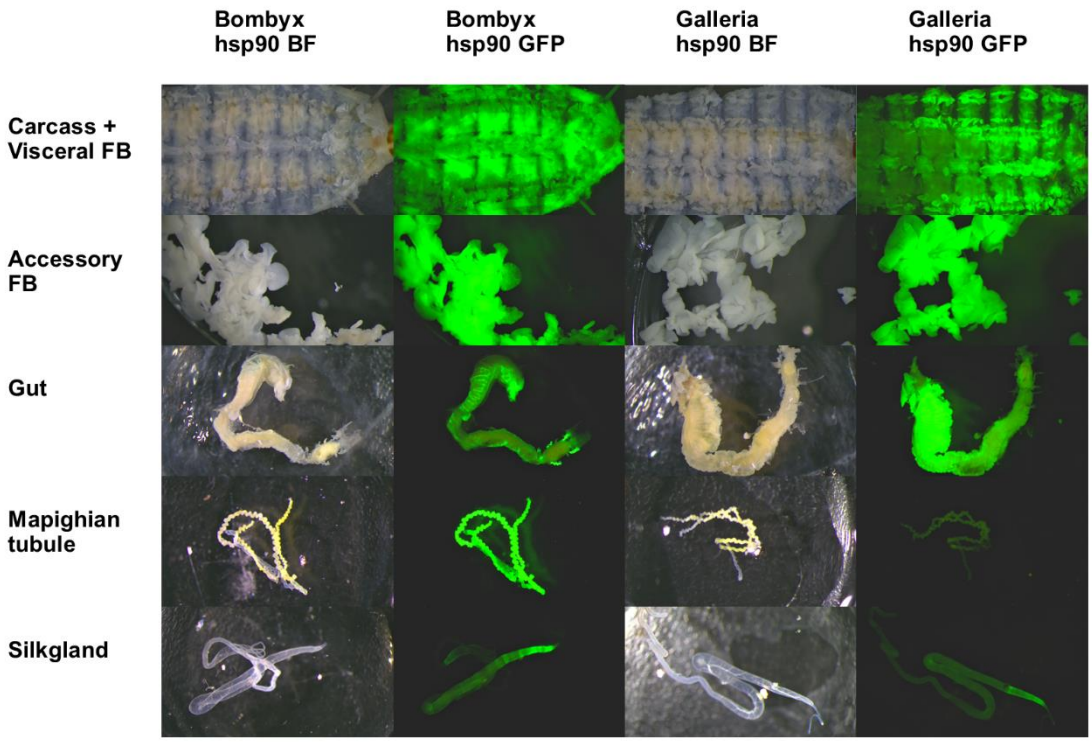

Figure 4.8 Variation between *Bombyx* and *Galleria* hsp90 promoter activity in *Galleria* tissues

Bombyx 2.9kb and *Galleria* 2kb upstream sequences of their respective hsp90 (hsp83) genes vary in their ability to drive GFP expression in *Galleria* tissues. BF and FB refer to bright field and fat body respectively. In the images of the carcass and the silk glands the anterior pole is to the right. In the *Bombyx* hsp90 line the GFP is unconjugated however, in the *Galleria* hsp90 line the GFP is fused to tubulin.

solely in the vitellophages, with GFP fluorescence then only visible once the pharate larvae was about to emerge through the chorion.

GFP- αTub1b and Histone-mCherry lines were crossed together to generate a line carrying both markers. This line was dimmer in both channels than either parent line, and showed a slightly different expression pattern for the Histone-mCherry fusion product than when the *Bombyx* hsp90 promoter drove GFP. No expression was observed in the Malpighian tubules and only very small patches of expression were seen in the silk gland (not visible in Fig 4.9). The changes in expression could be explained by insertion locus related expression profile, but this might also be due to the nuclear association of histone making detection and imaging more tricky due to at low magnification due to lower signal. It should also be noted that in general the signal to noise ratio in the red spectrum is much greater than in the green, since a large amount of *Galleria* tissue (and diet/food boluses etc.) is autofluorescent. This can make identifying signal from background noise at low magnifications tricky when imaging with dissecting microscopes with long pass GFP filters.

At higher magnification, under dissection microscopes, distinct nuclear expression patterns of mCherry could be observed in the fat body of GFP- αTub1b/Histone-mCherry larvae (Fig 4.10), however no GFP mitotic structures resembling microtubules could be observed at this magnification. At higher magnification using live embryos, it was possible to observe dense mCherry containing vitellophage nuclei. GFP containing structures that resemble cytoskeletal microtubules could also be observed but no spindle forming cells were observed that resembled those seen in the early embryo in fixed (Fig 4.11) or live tissue (supplemental material 4). Western blots against larval tissues from each parent strain revealed single bands for GFP at ~27kDa for the GFP strain and dsRed at ~40kDa for the Histone-mCherry

line (Fig 4.12). These accurately correspond to a monomeric GFP molecule and a Histone-mCherry fusion, suggesting that GFP may be cleaved from its tubulin fusion post translationally. This was surprising given that sequencing revealed the Tubulin moiety to be in-frame with the GFP molecule and no sulphur containing residues or other obvious targets for peptide cleavage are present in the linker.

Although described previously for other species it is worth discussing the 3xP3 expression profile in *Galleria*. Expression begins in late embryogenesis and can be observed through larval, pupal and adult stages. In larvae, brightest expression was observed from brain tissue and eye clusters but it can also be observed in all neural tissue with ganglia in each segment also particularly bright. The short length of this promoter makes it a great additional selection maker, but also useful general neural specific marker.

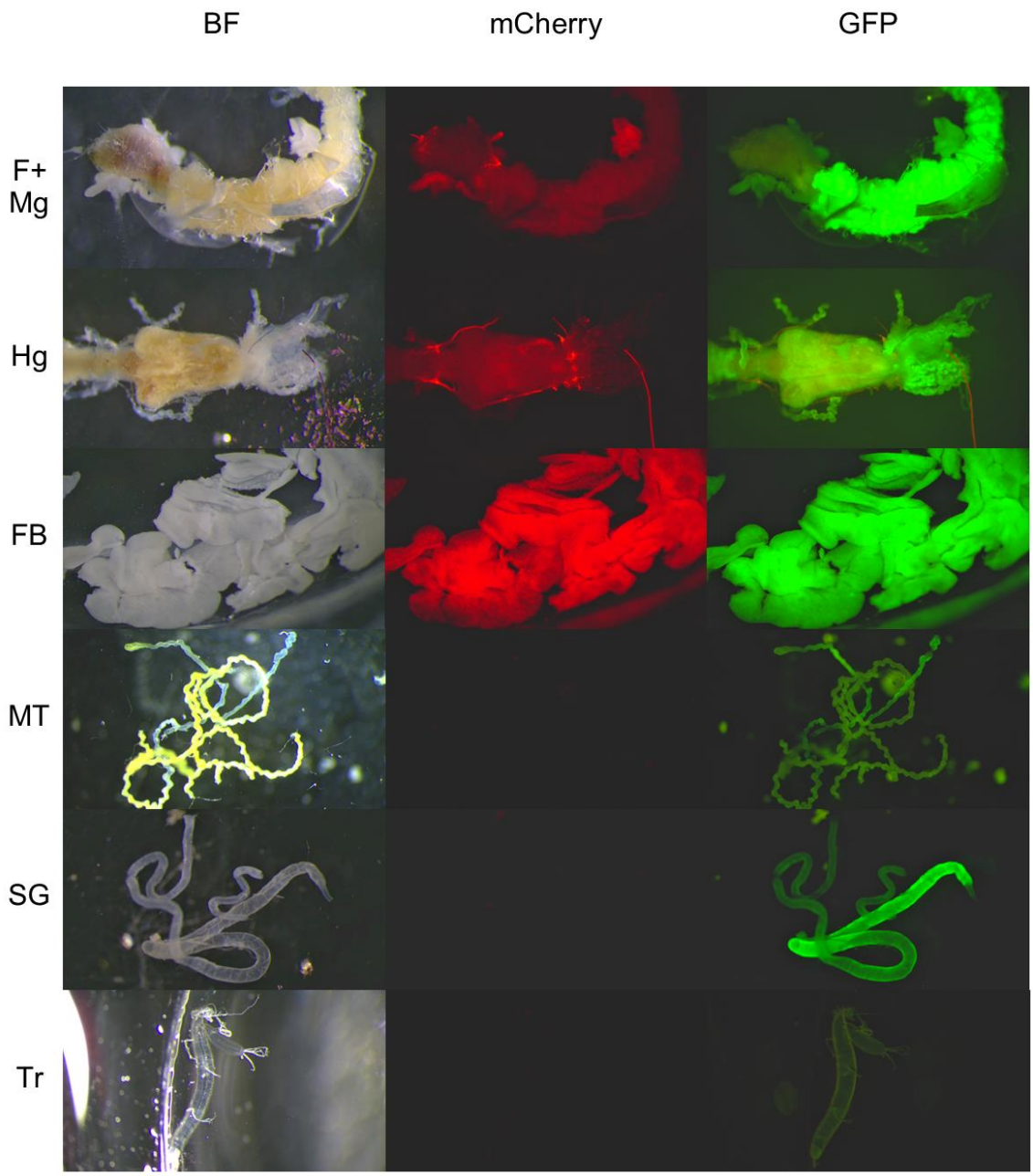

Figure 4.9 Reporter expression of GFP-αTub1b and His2av-Mch strains

The GFP-αTub1b and His2av-Mch fusion proteins, under the control of *Galleria* and *Bombyx* hsp90 promoters respectively, appear to show slightly different expression patterns to those seen with GFP expression. Noticeably HismCh is not expressed in the malpighian tubules or silk gland. F+Mg (fore and midgut), Hg (hindgut), FB (fat body), MT (malpighian tubules), SG (silk gland), Tr (trachea).

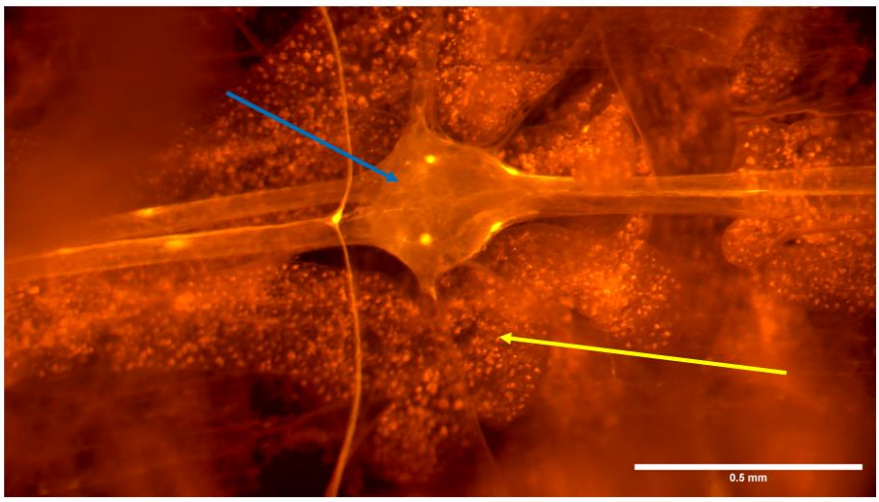

Figure 4.10 Nuclear type expression of mCherry in fat body tissue

Fat body (yellow arrow) and neuronal (blue arrow) tissue expressing mCherry and dsRed respectively in a dissected Histone2av-mCherry/P3:dsRed strain larva. mCherry expression within the fat body appears to be expressed in the nucleus

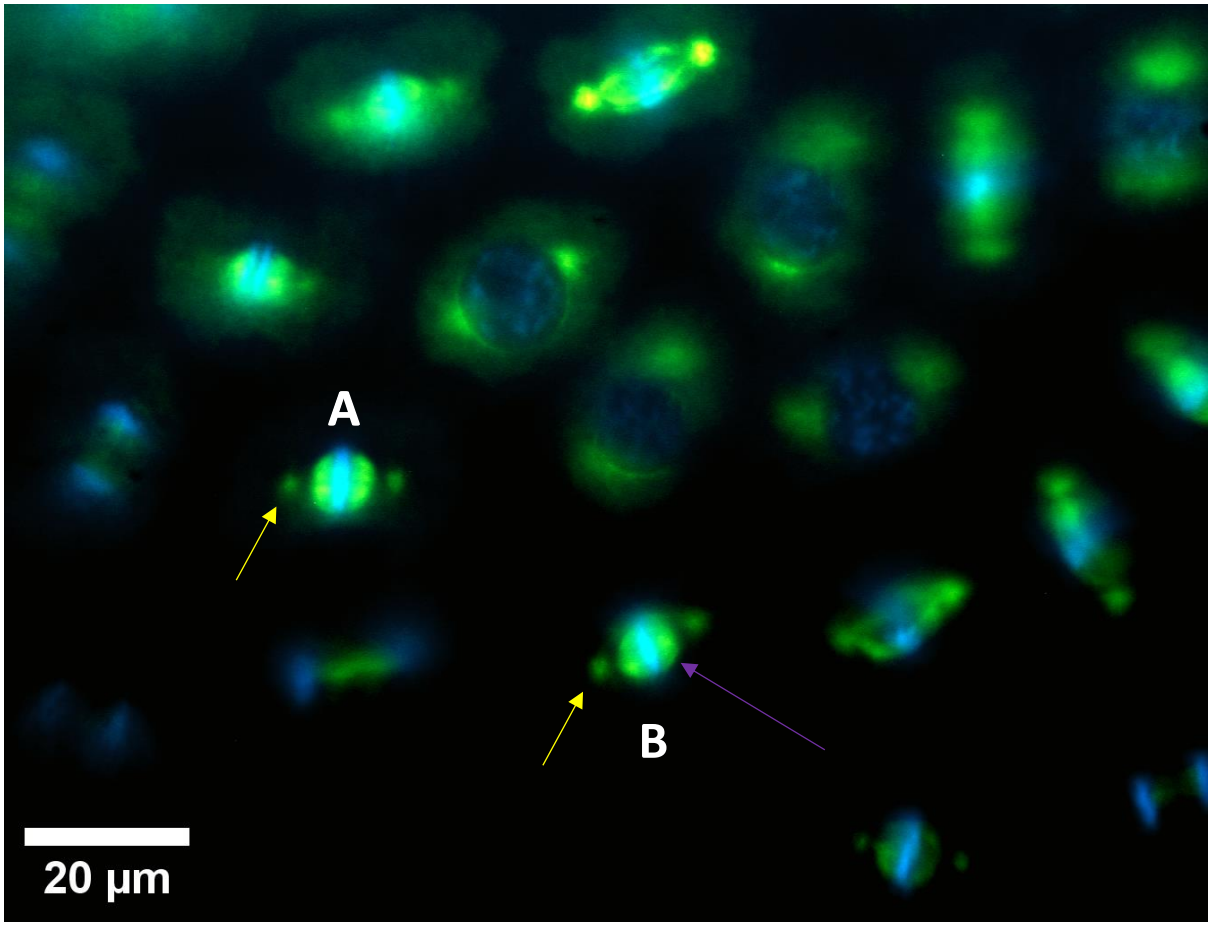

Figure 4.11 Mitotic spindles in the *Galleria* embryo

6-7hr old *Galleria* embryo stained with alexa-488 conjugated to an anti-tubulin antibody labelling α and β tubulin in green, with DNA labelled with Hoechst stain in blue. Centrosomes (yellow arrows) can be observed in metaphase nuclei (A and B) with a dense mitotic spindle (purple arrow) with separation between the spindle and centrosomes

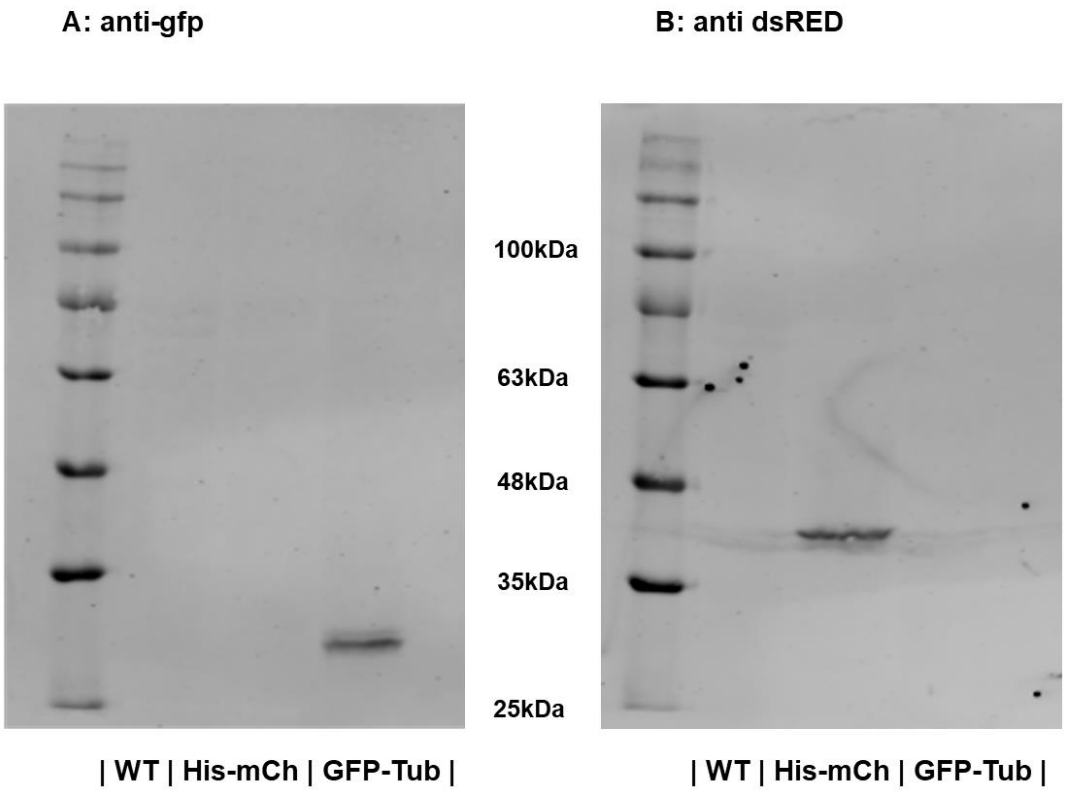

Figure 4.12 GFP appears to be postranscriptionally cleaved in GFP-αtub1b line

Separate western blots stained against GFP (blot A) and dsRed (blot B) run with larval tissue samples from wild type (WT), Histone2av-mCherry and GFP- αtub1b lines in three lanes from left to right. Whilst bands are present in both both lines as expected, the GFP band appear much smaller and resembles monomeric GFP in size (~27kDa) rather than the 75kDa protein expected. The ~40kDa band in the Histone2av-mCherry is the size expected.

4.3 CRISPR/Cas9 Mediated Mutagenesis

The method for utilising PiggyBac transgenesis to insert transgenes into the *Galleria* genome, described in the previous sections, is a fantastic tool for knocking in large and varied expression cassettes of interest. However, the ability to knock out genes of interest is also of great value and this is an area where PiggyBac transgenesis has it's limitations. The mostly random nature of PiggyBac mediated insertion means it is almost impossible to pre-determine which TTAA site the transposon cassette integrates at and therefore know which endogenous sequence it might disrupt.

Gene editing using CRISPR/Cas9 has developed massively in the last decade, and has been widely adopted for a variety of systems and applications. It is still most often used though as a very adaptable system for knocking out endogenous genes of interest.

I sought to adapt this technology for the *Galleria* system, using the GFP CDS in our newly created transgenic strain as an initial target for proof of concept. The *Bm*hsp90:GFP/3xP3:DsRed provided a perfect target for this as the GFP and RFP expression provides dual dominant selection markers for screening and the known target sequence and flanking regions compensate for an imperfectly assembled and annotated genome. The knockout phenotype was anticipated to be disrupted GFP expression and therefore be very visible in G0s, enabling easy assessment of CRISPR induced mutagenesis. The dominant RFP marker would still be present if G0s were outcrossed to WT individuals and would demonstrate that the injected G0 individuals were homozygous for the transgenic construct and that a mutation in the GFP CDS was indeed present.

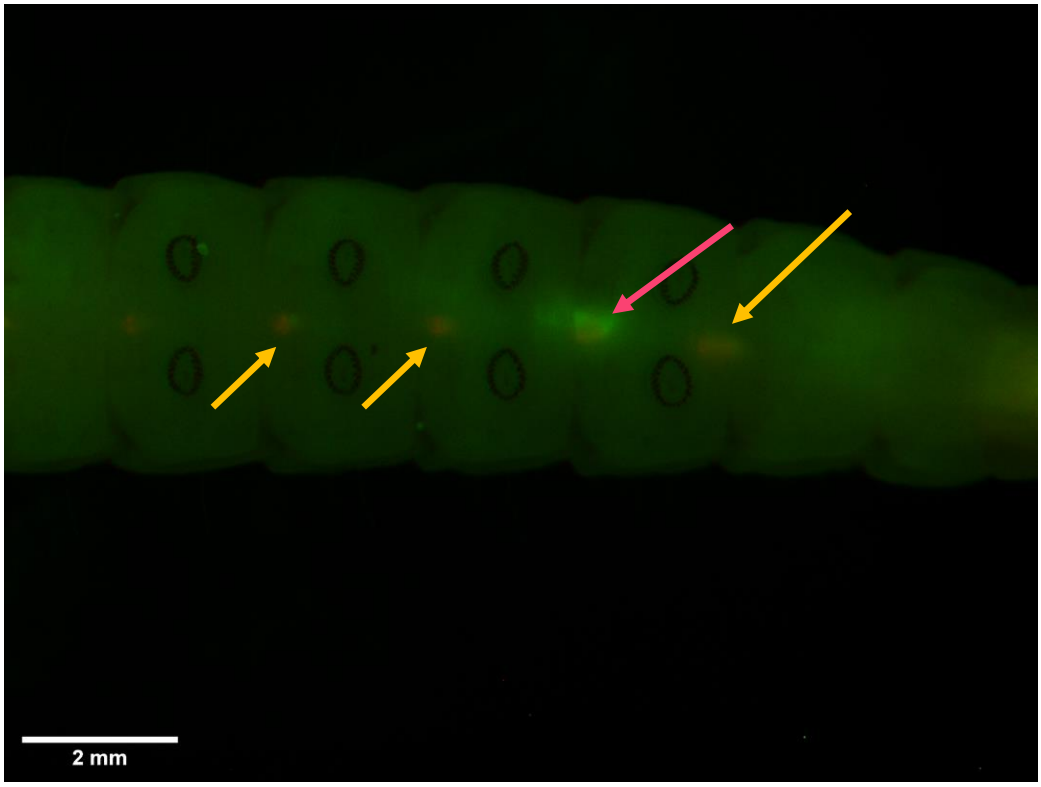

Figure 4.13 CRISPR/Cas9 mutagenesis is able to strongly disrupt GFP expression in a G0 larva

Posterior dorsal view of Cas9/sgRNA RNP injected *Bm*hsp90:GFP/3xP3:DsRed G0 larva, with almost total somatic gene knock-out of EGFP gene. Foci of neuronal RFP expression can be seen at the intersegmental junction (yellow arrows) with a small section of EGFP expression remaining (pink arrow).

A visit to the Mosimann lab, in Zurich at the time, demonstrated both the accessibility and ease at which genomic indels could be created using *in vitro* assembled ribonucleoprotein complexes (RNPs) in the zebrafish model organism and in Insect models by their collaborators. I sought to apply this technique to the *Galleria* system using a sgRNA against a sequence towards the N terminal of the GFP CDS previously described by Jao et al.[197] Personal correspondence with the Mosimann lab indicated that, in zebrafish, this particular sgRNA induced cuts at the target sequence with high efficiency.

sgRNA/Cas9-mCh RNP mixes were assembled *in vitro* as described by Mosimann[198] and injected into 1-2hr embryos as previously described in chapter 3. Hatch rates were similar to those observed in PiggyBac mutagenesis experiments at 30.5% but pupation rates were lower at 7%. It was anticipated that both these might be higher since protein and mRNA were thought to be less toxic as they are degraded quicker than plasmid DNA. However, the high sgRNA concentration used and possible off target cuts within the genome may have led to increased mortality.

Compared to PiggyBac methods though, a large proportion of the G0 larvae showed a transgenic phenotype. In larvae this ranged from small patches of GFP negative tissue and reduced overall fluorescence intensity to almost complete absence of GFP expression (Fig 4.13), but otherwise larvae appeared healthy and developed normally. It is clear therefore that this technique, as in other organisms, is a powerful tool for inducing loss of function mutations in targeted sequences in G0 individuals.

To assess the heritability of these mutations, G0 injectees were crossed to wild type individuals and the progeny screened for a knockout phenotype. All progeny possessed red eyes, indicating that all G0s were homozygous for the transgene and

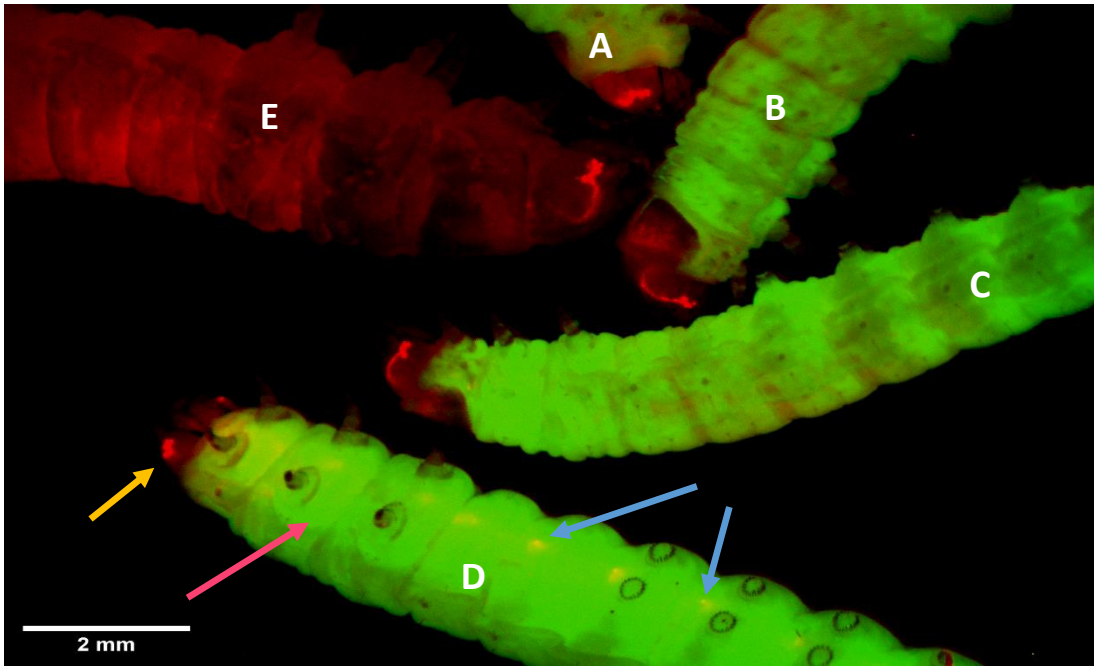

Figure 4.14 CRISPR/Cas9 is able to induced heritable mutations to the GFP CDS

(A-E) Transgenic larvae of the waxworm moth, *Galleria mellonella*. Bmhsp90:GFP/3xP3:DsRed G0 larva A-D have an expression cassette inserted into their genome resulting in expression of EGFP visible in all somatic un-melanised tissue (pink arrow) and RFP in eye (yellow arrow) and neural (blue arrow) tissue. Larva E is the G1 offspring of individuals of the same transgenic line as A-D but injected with a Cas9/sgRNA ribonucleoprotein targeting the EGFP gene, causing a heritable loss of function in the GFP gene but retaining the eye specific RFP expression

that any knockout phenotype observed in G0s was biallelic. 14 out of 28 crosses produced progeny with GFP -ve phenotype, ranging from the entire brood to just a single knockout individual. Interestingly, G0 injectees that showed no visible change in phenotype produced knockout offspring possibly indicating that G0 somatic phenotype does not necessarily correspond to the G1 phenotype. G1 individuals were then sibling mated and a homozygous line maintained for 5 generations with the same phenotype – indicating that these heritable mutations are stable.

To assess the types of mutations that this method created within the GFP CDS, gDNA was extracted from a sample of knockout G1 larvae from different crosses and the N terminal GFP CDS amplified using primers GFP-F and GFP-R. Sequencing these PCR fragments revealed various different CDS mutations including deletions, small insertions and substitutions (Fig 4.15), consistent with those reported in other organisms.

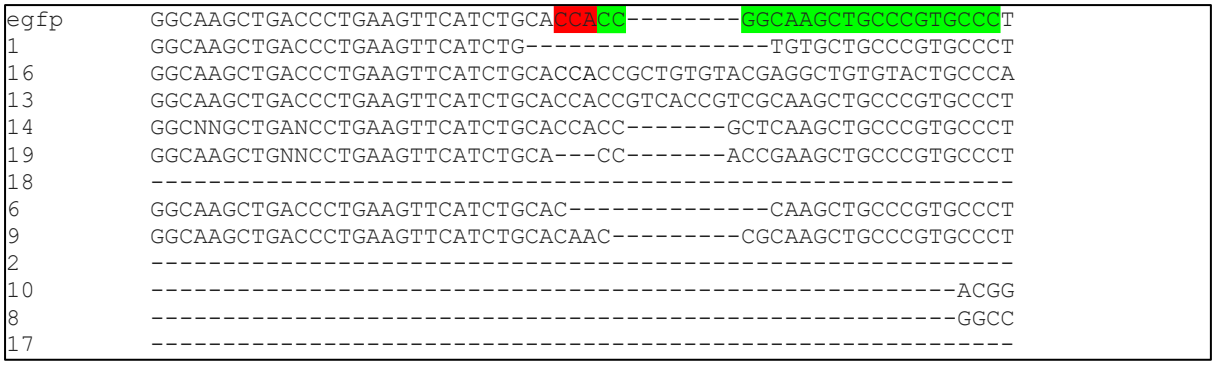

Figure 4.15 CRISPR induced mutations in genomic EGFP CDS in G1 *Bm*hsp90:GFP/3xP3:DsRed larvae

Mutations within the coding sequences of 12 different GFP negative G1 crispants. The G1 larvae were offspring from individuals crosses of Bmhsp90:GFP/3xP3:DsRed G0s injected with an sgRNA targeting the N-terminus of the eGFP sequence, and crossed to wild type mates. The top line is the native eGFP sequence with the sequence complementary to the sgRNA highlighted in green and the PAM sequence highlighted in red.

4.4 Discussion

This chapter presents a demonstration of novel techniques for this organism, which have allowed both insert the insertion of new genetic material and generation of loss of function mutations at existing endogenous loci. It is a clear demonstration that the techniques are a viable method for creating transgenic *Galleria* and removes one of the main obstacles to further uptake of this model.

PiggyBac transgenesis is a useful tool, allowing genetic cargos to be hopped semi-randomly into the genome. It is not, however, without its issues as it inserts seamlessly into TTAA repeats and has a preference for open chromatin which increases the likelihood of inserting into coding regions. In addition to this, PiggyBac mediated mutagenesis tends to have low insertion efficiencies, as seen with *Galleria* in this chapter, and survival rates tend also to be lower due to toxicity from plasmid DNA. This then requires large numbers of embryos to be injected, however the observed hatch rates, pupation rates and proportion of offspring that are transgenic appears to be within ranges seen for other organisms and new stable transgenic lines are achievable within 6 months. Its main benefits however are its abilities to insert large cargos (>10kbp) and the ease and reliability of the technique.

In *Bombyx* the helper pHA3PIG is widely used, and has been successful in generating transgenic offspring in not only a number of different *Bombyx* laboratories but also different lepidopteran species[214]. It is therefore surprising that it appeared to generate very little transposase activity in *Galleria* despite using the same parameters that worked well in *Bombyx*[77]. Transgenesis efficiency in *Bombyx* using this technique was greatly increased by refining the injection location within the embryo, ensuring that the injection droplet colocalises with the presumed location of

the primordial germ cells. In *Bombyx* it is possible to roughly identify this posterior-ventral from markings on the embryonic chorion, however this has not yet been possible in *Galleria* using low powered microscopy.

The use of mRNA vs DNA transposase helper could improve the efficiency of this technique, and hasn't been fully explored in this organism. A set of injections was done with a hyperactive PiggyBac mRNA transposase, and several mosaic transgenic G0 observed, but no transgenic G1s resulted. It is likely that if more injections had been done, that transgenic offspring would have resulted and suggested that the mRNA transposase is similar in efficiency to the DNA transposase. The advantage of the DNA transposase has in terms of easy propagation, molecular stability and cost though, means though that any theoretical advantage is likely outweighed.

In truth it was frustrating that despite trying two different hsp90 promoters, no transgenic strains with embryonic expression were developed. The *Bombyx* hsp90 promoter showed strong activity in embryonic tissue (ref) so it was surprising that it was only active in the vitellophages in *Galleria* but not the syncytium or embryonic germ band. No embryonic expression was observed with the *Galleria* promoter despite being active in larval and adult tissue. The shorter 2kb upstream sequence used compared to the 2.9kb used in *Bombyx* may be responsible for this, as it may encode embryonic specific promoter elements. In comparison with the *Bombyx* promoter, there are also less canonical heat shock elements in the first 2kb of the *Galleria* upstream region, with several found in the 2-3kb region. Missing out these HSEs from the promoter could explain the reduced brightness seen in transgenic lines and also the non-uniform expression throughout the body.

Of the two fusion lines generated, only the histone-mCherry line created has a consistent phenotype with regards to both the spatial distribution of fluorescence within the organism and the cellular localisation of the fusion proteins. The GFP-αTub line has shown variations in brightness and location of fluorescence in larvae between generations and an inconsistent localisation within the cell when observed at higher magnifications. Western blotting confirms that in some individuals the GFP moiety seems to have dissociated from the Tubulin, however microtubule like structures can be observed in others. The variability in the GFP-αTub overall brightness may also be due to differential regulation of the transgene by the promoter in response to life cycle events (ecdysis, pupation etc.) or reaction to other biotic and abiotic stressors. Whilst hsp90 has been shown in other organisms to not be as highly upregulated in response to stress compared to other hsps it does still show some variability in its expression. When the two strains are crossed together, the hybrid individuals tend to have reduce brightness, which might indicate that in co-expression of the two proteins the promoters are competing for upstream regulators or that supporting expression at such a high load has deleterious effects on their fitness. Overall though, whilst it would have been ideal to have strains that co-expressed these two proteins constitutively across the organism and all its life stages by using the same promoter and landing site, this works as a good proof of concept for future strains.

CRISPR/Cas has now been shown to work in a wide variety of models, including Lepidoptera, and has been adept at generating indel mutations, large deletions and short insertions. Adapting the CRISPR/Cas9 system to *Galleria* was surprisingly easy, once the microinjection protocol had been sorted and after training from the Mosimann group. The previous success in adapting the Mosimann group protocol for

RNP assembly and delivery to Musca domestica[157] and Ceratitis capitata[215], which is regularly used by their lab for inducing mutagenesis in Zebrafish played a large part in this quick adaptation.

The hatch rates and pupation were comparable to some of the PiggyBac experiments from this chapter, however they appear very low compared to similar CRISPR based experiments in other organisms. It is likely that this was due in the main part to the high concentration of guide RNA used, which at 2µg/µl was outside the range of 50-300ng/µl l commonly used in other organisms. It is possible that off target cuts were also responsible for the increased mortality, as search of the *Galleria* RefSeq genome using the CRISPOR[216] web interface revealed 10 off targets within that assembly. However, none of these off targets had less than 4 mismatches and none had no mismatches within the first 12 bases in the 3' section of the guide. This was manually repeated by BLASTing the guide against the Korean *Galleria* assembly[68] as in practice our lab has found significant differences between the 3 *Galleria* assemblies, but again no likely off target cuts were found.

In comparison with PiggyBac transgenesis, what was evident was the much higher rate of mutagenesis. A large proportion of G0 larvae showed a mosaic phenotype, some with almost complete loss of GFP expression, and half the G1 broods contained GFP negative larvae which have retained their mutant phenotype stably over several generations. Whilst these results show that CRISPR/Cas is indeed able to successfully generate mutant phenotypes at both G0 and subsequent generations, it should also be noted that these results are from initial experiments. As the protocol is optimised further, with regards to timings and injection mix concentrations, it would be expected that not only increase efficiency but survival will

increase too. This is an area of *Galleria* research with massive future potential and countless possible areas for expansion.

Generation of transgenic tools for *Galleria* was a core aim for this project, and widely seen as crucial for unlocking the potential of this model as a replacement for rodent infection models. As these molecular and genetic tools progress and more researchers incorporate the model into their work, *Galleria* will be able to be used much more widely than the current focus on microbial pathogens.

Chapter 5

Discussion

In this thesis project the primary goal was to advance the available suite of tools in *Galleria*. By developing a robust protocol to inject embryos and optimising genetic techniques such as PiggyBac and CRISPR in this system, a template would be available for researchers looking to create their own strains of *Galleria*. The ability to create specific genetic backgrounds would then vastly increase the number of applications as a rodent replacement model in studies on infection, immunity and inflammation. In this chapter the level of success to which this goal was achieved, what more information is needed and how the finding presented within the thesis could be taken forward.

Optimisation of a *Galleria* rearing protocol

The rearing method presented incorporates various elements from different published methodologies [3,5,6,60] and has adapted them for production of large numbers of embryos within a non-specialist space in a lab environment. Large numbers of all other life stages can be produced which may benefit researchers also looking to use larvae, with very low proportions of batches falling sick or failing to develop.

It works particularly well for unsupported researchers given that it takes little space, requires few specialist reagents or materials and both time and monetary costs are low with ~two hours per week spent on maintenance time and ~£500 per annum on consumables. The vast majority of rearing materials are reusable and long-lasting saving on equipment costs and lessening environmental impact.

Some issues do remain however, relating to containment of L1 larvae. Despite best efforts the smallest larvae which hatch in adult jars from uncollected embryos are able to crawl around threads in jar lids and escape into the incubator. This could lead to cross contamination between strains and also potentially raise containment issues if they were to escape given they are natural pests of vital pollinators. Potential solutions to this include using ringed layers of petroleum jelly on the outside of jars to stop escape or by using larger versions of the foam bungs which appear to deter L1 larvae kept in small *Drosophila* tubes. These bungs could be protected from damaging gnawing by older larvae by placing steel mesh between them and the jars provided there was a snug fit. For the larger part, containment did not appear to be a problem as escaped L1 larvae remained within the warmer environment of the incubator and quickly desiccated, however bait petri dishes containing uncovered diet were placed near all larval and adult jars to attempt to mop up any escapees.

Investigations into the timing and patterns of early development

Imaging of temporally staged and fixed embryos during early development provided a view of the pattern and timings of energids migration. This information is very useful in the context of embryonic microinjection for different transgenic methodologies, providing a time limit by which injections must be complete to successfully direct mutagenesis at a single cell stage or before cellularisation (depending on the technique).

The images seem to suggest that a three-hour window post oviposition is ideal for injecting, corresponding to roughly a 1-2 nuclei stage, with the blastoderm forming by 6-7 hrs at 30C. This broadly correlates with timings observed by Abidallah[3]. To gain

a more practically relevant understanding of the timings it could be worth repeating this but using the storage methods normally employed during microinjection protocols (30C for 1hr followed by 18C storage). It appears that *Galleria* develops at a similar speed to other Lepidopterans, although this is likely to be temperature dependent, as in both *Bombyx* and Manduca it has been reported that blastoderm formation takes around 10hrs to complete at 29C and 24C respectively [217,218]. Drawing conclusions on spatial patterning of energids in the early mitotic divisions in *Galleria* embryos is difficult using the current methodology as embryos were squashed to varying degrees under the cover slip which could distort energid localisation.

In these results, loss of synchrony in divisions was used as a marker for cellularisation, as synchronous waves of division are maintained by cytoplasmic flow and control of regulators that is only possible when sharing a common cytoplasm[219,220]. Unfortunately, IHC methodologies to determine the presence of membranous barriers at the cortex using antibodies raised against *Drosophila* failed to show any specific binding. Confirmation of cellularisation would be useful and could be done via a variety of methods including staining with membranous dyes such as CellBrite or via injection of mRNAs early in development. Co-injection of dual mRNAs encoding endogenous Histone proteins and a membrane marker such as Pnut (*Galleria* Septin-7, LOC113518577) that localises to the cortex in *Drosophila*. This would have the added benefit that development could be tracked via the histone marker using time lapse imaging and give more detailed information about precise developmental timings.

Developing a microinjection protocol for *Galleria*

The microinjection protocol for *Galleria* presented here compares well with other lepidoptera in terms of post-injection survival rate [110,221,222], although data is limited for control injections in other species such as *Bombyx*. Removal of the chorion helps prevent needle breakage and does not appear to impact survival greatly, allowing other applications such as live imaging. Provided they are dechorionated properly, *Galleria* embryos are robust enough to survive physical manipulation pre and post-injection. The process of lining up embryos against slides is time consuming but seems to result in successful injections and, as long as adequate humidity levels are maintained, larvae have no issue developing on glass slides and traversing their way over them and petri dishes to find diet once emerged.

One major issue still remaining with this microinjection protocol stems from a limitation in our understanding of *Galleria* embryo morphology. Neither *Galleria* embryos, nor their chorion are pigmented and apart from their micropyle very little information is available to determine anterior-posterior and dorso-ventral axes. In young embryos, when the chorion is removed embryos become even less defined in their shape and no internal structures such as cytoplasmic islands exist[3] to be able to orientate them. This has implications for transgenesis efficiency as Tamura et al.[109] showed that PiggyBac donor integration could be greatly increased by injecting into the area though to encode the primordial germ anlage, determined by the aggregation of nanos transcripts[199]. This is possible in *Bombyx* because the eggs display patterned chorions which enable localisation of this area, and the microinjection techniques do not require removal of the chorion. Localisation of this tissue should be a priority in *Galleria*, which could initially be achieved through fluorescent *in-situ* hybridisation assays but more useful would be reporter lines under

the control of nanos promoters to enable visualisation and orientation of embryos during plating.

Creation of a transgenic reporter line

The generation of a stable reporter line in *Galleria* is a major step forward, and follows the template seen in many other insects, utilising PiggyBac transgenesis and a combination of DNA based donors and transposases to mediate mutagenesis in the insect germ line [77,110,111,209,214,223–226]. The use of fluorescent reporters allows rapid and screening of phenotype, as the distinct differences to wild type fluorescence can aid detection when promoter activity is less than expected[77].

For injections containing donor and helper plasmids, hatch rate success was similar or slightly lower to those described in experiments involving *Bombyx* and *Plutella xylostella* [77,109,209]and comparable or better than other diverse insect species[227]. This is likely strongly influenced by microinjection technique and methodology, as well as the individual skill of the operator and will likely improve with practice.

In *Galleria* the helper pHA3PIG, which has been used to generate numerous transgenic Lepidoptera was unsuccessful in generating germ line integration and only generated somatic integration extremely rarely. It is possible that this is due low activation of transcription by the *Bombyx mori* actin 3 promoter within the embryo or low activation of the native PiggyBac transposase within the embryo. Of the two, low transcription inducing activity of the actin promoter is likely given that in *Bombyx* it was not very active in embryonic germ tissue [228] and native PiggyBac has been shown to be active in other Lepidoptera.

The reporter used to successfully generate transgenic *Galleria* contained dual fluorescent markers that had both been described have strong expression profiles in closely related species[204]. GFP was driven by the *Bombyx* hsp90 promoter and in *Bombyx* this had much higher activity in all life stages compared to the actin. Strong expression driven by the *Bombyx* hsp90 promoter was also seen in *Galleria*, however it was absent from embryonic germ tissue until the pharate larvae had formed. This made screening more cumbersome as larvae had to be allowed to develop until large enough to handle. It also has implications for transgenesis efficiency as the *Bombyx* hsp90 promoter was also used to drive the transposase.

Key to future work therefore should be developing transgenic constructs that drive strong expression in the embryo as well as larval and adult tissues. Not only would this decrease the workload needed for screening, but it could also lead to greatly improved transgenesis efficiency as genetic cargo could be incorporated into tissues at a much earlier developmental stage. Likely candidates for potential strong constitutive promoters would be the polyubiquitin promoter, which is used widely used in *Drosophila* or α-tubulin promoter, both of which have been successfully used in Tribolium[224,229].

Creation of strains expressing fusion proteins

The premise behind creating transgenic lines expressing fusion constructs, beyond just proof of principle, was to deliver co-expression of proteins that would enable visualisation of cell division in live tissues. This aim was unsuccessfully delivered during this project, due to several factors.

The primary issue was due to the activity of the promoters used to drive expression in either strain. The GFP-αtubulin1b reporter fusion protein was driven by a shorter endogenous *Galleria* hsp90 promoter, in an effort to reduce construct size, which showed weaker expression compared to the *Bombyx* hsp90 promoter. This promoter was inactive or weakly active in embryos, certainly compared to the results reported by Tsubota[204], and unfortunately did not closely overlap in expression pattern with the *Bombyx* hsp90 promoter used to drive Histone2AV-mCherry expression. The second issue was variability in expression between generations and a incongruence between what was observed under low power or confocal microscopy. Western blots seem to suggest that most of the GFP-tubulin fusion product was cleaved post translation, although microtubule-like cytoskeletal structures could be observed in embryos.

Ultimately it was not possible to observe microtubule dynamics using these constructs, however it would be a suitable system for testing new constitutive promoters. Replacement of hsp90 promoters in either plasmid with polyubiquitin may solve the lack of embryonic expression and overlap between expression locations. It is likely also to be wise to redevelop the linker used to fuse GFP to α-tubulin given its evident cleavage.

There are some benefits from the results of this experiment though. The ability of Histone2av-mCherry to localise to the nucleus indicates that in *Galleria*, fusion constructs retain some of their function and are able to bind DNA. Despite overexpressing proteins that play key roles within the cell they were tolerated well with no observable loss of fitness. Tubulin and histone are both abundant within the cell however, so it could be possible that overexpression of naturally less abundant proteins have more deleterious effects. Measures of fitness were not quantified in

this study, however for future strains this could be investigated further via hatching, pupation and fertility rate assessments along with notation of any morphological deformities or sickness.

Further development of resources for generating reporter lines is needed, as a library of fusion constructs known to work would be greatly beneficial to researchers looking to monitor their genes of interest *in vivo*. Of particular interest may be genes associated with immune response which would enable tracking of infection response whilst the animal is still alive such as *hemolin* or *apolipophorin III*, thus reducing the number of larvae needed experiment whilst also gaining more information per experiment.

CRISPR mediated gene knock-outs

The use of CRISPR in this organism is another major advance in the *Galleria* toolset. The ability to use transgenes such as GFP, already present within the organism's genome, as genomic targets provide a dominant screening marker and reduced time needed to optimise guide sequence as efficient guides could be chosen from the literature[197].

CRISPR is an extremely promising technique for targeted mutagenesis in *Galleria*, given its ability to induce loss of function in large proportions of G0 somatic cells and in the next generation. In the experiments conducted, hatch rates and development to pupation were low, however this is almost certainly due to the concentration of guide used[146], which is much higher than other values cited in the literature [147,230]. In *Galleria*, this technique would benefit from refinement in this area to determine the trade-off between cutting efficiency and mortality at different concentrations. Ideally

this will be done with both user-made and commercially produced guides, to detect any differences and ensure maximum reproducibility for other groups.

The next step will be to target endogenous genes. In other lepidopterans, genes with a visible phenotype were targeted [155,231–233]such as *white*, *yellow* or *ebony* loci, of which orthologues are present in *Galleria*. The visible phenotypes aid detection of G0 and G1 individuals bearing mutations and genotyping is not required to spot crispants. Targets that are not likely have a visible phenotype will likely involve lengthier molecular based techniques to identify crispants, reducing throughput, however there are methods that reduce the effort required. These include targeting restriction sites or using two guides to generate small deletions so genotyping can be achieved by PCR based assays, or a strategy known as co-CRISPR where dual guides are used a visible marker concurrently [234]. There are also knock-in based techniques such as CHoP-In or CRISPaint[235,236] which cause loss of function but introduce dominant markers via NHEJ to allow rapid screening. The ability to generate new loss of function strains in the future would have plenty of applications that benefit researchers and may be of particular use to those investigating host-pathogen immune interactions. Instead of only mutating microbial virulence factors, researchers would also be able to modulate the host immune response via targeting elements of Toll, IMD or PPO pathways.

Where there is most promise for CRISPR in animals, is as a tool for targeted integration of new sequences. Short knock-ins using ssODNs are currently the most efficient methods, however knock-in methodologies that enable longer sequences to be inserted are beginning to be widely used. At the moment though, longer CRISPR knock-ins seem to have varying success depending on the repair method, organism, nuclease source and repair template [237–239].

Future directions

There are multiple ways that findings in this thesis could be immediately used to advance our understanding and scope of use in *Galleria*.

The first would be use transposase based techniques to generate strains that use polyubiquitin promoters to drive reporter constructs, and investigate their ability to drive constitutive throughout all tissues. The reporter constructs used could again be histone and tubulin fusions. This would provide the dual benefit of allowing visualisation of dynamic mitotic processes during early development but also provide detailed a reliable nuclear, cytoplasmic or cellular marker for infection studies on haemocytes where flow cytometry is often used to analyse immune cells.

The second would be to generate reporter lines that mark the germ line in embryos. *Nanos-O* transcripts have been shown to localise to germ tissues in all life stages of *Bombyx* (Nakao 2008) identifying the promoter of this gene as a strong candidate. This could also be coupled with Cas9 expression, which would then provide endogenous nuclease activity within the germline for CRISPR based techniques whilst also acting as a signal for the location of injections for DNA plasmid mediated transgenesis methods.

Cas9 expressing embryos, along with WT ones, could then be used to optimise CRISPR knockout protocols and attempt various knock-in strategies. Targets for knock-outs could include PRRs or proteins involved in opsonisation such as haemolin or apolipophorin III, to see what effects these have on different pathogen classes. For knock-ins it would be particularly useful to be able to fluorescently tag genes that are known to be upregulated in response to infection, such as cecropin D

or Hdd11[240]. Not only could these be used to monitor the strength of immune response during the course of an infection but it could also be used to detect baseline health before assays.

Summary

To conclude, the work described in this thesis has advanced the scientific body of knowledge with respect to *Galleria mellonella* in several ways.

It has described a method by which foreign materials may be introduced into the early embryo without causing excessive lethality, and the timings during which this must be done to ensure uptake of material upon cellularisation. It has also described a method by which PiggyBac transposase may be used to introduce exogenous genetic material into the genome and demonstrated that this can be used to express fusion constructs which localise to particular structures within the cell. Lastly it presents a method by which CRISPR/Cas9 ribonucleoprotein complexes can be used to disrupt gene function within the organism in a heritable manner.

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