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Informed Development of a Flystrike Vaccine

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Contents

Executive Summary

Our analysis of the variation in the genetic composition of individual flies sourced from the third and final round of population collections conducted in the 2020/2021 season has been completed. The results have been consistent with prior years, clearly demonstrating the samples clustering as three distinct populations. We have discovered that there is a strong correlation between the geographic location of the collection sites and genetic differences between these populations indicating a strong signal of the isolation of populations by distance. We have also discovered the presence of gene flow/migration occurring between flies in regions along the East coast of mainland Australia. Given the relatively close proximity of sheep farming properties that range from southern QLD down the east coast of Australia through to VIC and across to SA, this is likely to be a major factor driving gene flow that we observed between these populations.

The multi-year combined analysis has now been completed. The data set includes 160 sample collections and 2915 individual *L. cuprina* flies. Across the project, the sample collections have been able to cover major sheep growing regions of WA, VIC, NSW, SA, QLD and TAS. Based on our analysis we have been able to determine that there are three distinguishable population clusters in Australia. One in Western Australia, a second population in Tasmania and the third is a large Eastern Mainland population that covers samples from regions spanning QLD, NSW, VIC and SA.

The population data will be an important resource for both understanding the spread of chemical resistance and assessing feasibility of area-wide control strategies that may be available in the future. We are working to disseminate this information and an important outlet is through peer-reviewed scientific journals. A manuscript detailing the mitochondrial genomes of *Lucilia* spp. from QLD (*L. cuprina cuprina*), NSW, VIC and WA (*L. cuprina dorsalis*) and TAS (*L. sericata*) has been drafted. A second article on the Australian blowfly population structure has also been prepared.

In addition to the direct findings of the study, it will be important for this information to be captured for future use by industry and other researchers investigating methods to control the Australian sheep blowfly. To this end we have recommended the following: that the AWI develops strategies that will improve the retention and access to this and other study data so that it is readily accessible to interested parties, that the population data is of significant value and should be integrated and further analysed as part of future insecticide resistance studies and that the novel vaccine candidates identified in this study be available to researchers thereby ensuring that ongoing value can be extracted from this important genomic and proteomic resource created in this project.

The genomic analysis from nation-wide sampling of 2,915 *Lucilia cuprina* samples from 160 collection sites over three collection periods (2019, 2020/21 and 2021/22) uncovered significant genetic variation in the Australian sheep blowfly population. Using individual DArTSeq and Pool-seq methodology to compare similarities and differences in genetic information from individual flies we were able to genetically discriminate three distinct blowfly populations in Australia. Flies collected from Victoria, New South Wales, Queensland and South Australia are genetically similar to each other and interbreed, forming one large population. Those flies found in Western Australia and Tasmania form two other distinct populations of blowflies.

The level of correlation between the geographic location of the collection sites and genetic differences between these populations strongly signals the isolation of populations by distance. We have also discovered the presence of gene flow/migration occurring between flies within regions along the East coast of mainland Australia. Given the relatively close proximity of sheep farming properties that range from southern Queensland down the east coast of Australia through to Victoria and across to South Australia, this is likely to be a major factor driving the gene flow that we observed between these sub-populations.

Results from the genomic analysis also suggested no evidence of genetic changes that would be associated with target site resistance to imidacloprid or spinosad, two insecticides currently available for protection or treatment of flystrike.

However, resistance alleles that have previously arisen to older chemicals, dieldrin, malathion and diazinon were identified in some of the population samples during the study.

This information will be an important resource for understanding the spread of chemical resistance and developing regionally targeted woolgrower management strategies to protect and sustain the effectiveness of current blowfly chemicals, as well as for assessing the feasibility of new blowfly control strategies. We are working to disseminate this information and an important outlet is through peer-reviewed scientific journals. A manuscript detailing the mitochondrial genomes of *Lucilia* spp. from Queensland (*L. cuprina cuprina*), NSW, Victoria and WA (*L. cuprina dorsalis*) and Tasmania (*L. sericata*) has been drafted. A second article on the Australian blowfly population structure is also being prepared for publication.

Another area of this project was a study that aimed to understand how flies establish a strike, particularly what proteins are critical in the early stages, just prior to and during the initiation of a wound. Sampling from an *in-vivo* implant trial, when blowfly maggots were starting to create a wound, identified hundreds of fly proteins that are being excreted during strike initiation, providing detailed insights into this complex biological process. These results are contributing to our understanding of the way sheep respond to strike and the battle between the maggots and sheep defence mechanisms.

The analysis revealed the presence of several proteins that are of interest to CSIRO and their program to develop a vaccine against flystrike. We also compared highly expressed genes from a previously generated blowfly transcriptome Perry and Bowles (unpublished) to those proteins identified in the current study using proteomics. This approach has validated proteins that we had previously predicted to be secreted during the early stages of maggot development as well as identifying a range of additional proteins that had not been identified using gene expression data but were found to be present and at relatively high levels during maggot development. These studies are assisting us in our efforts to identify further candidate proteins that may contribute towards the development of therapeutics, such as vaccines or novel chemicals, that could assist in protecting sheep against flystrike.

In addition to the direct findings of the study, it will be important for this information to be captured for future use by industry and other researchers investigating methods to control the Australian sheep blowfly. To this end we have recommended the following:

- Develop and implement a strategy to preserve the data and resources from this project as a readily accessible resource for future studies.
- Integrate the genomics and population data with future insecticide resistance studies and conduct further analyses to extract more value from these valuable large multi-season datasets.
- Make the novel vaccine candidates identified in this study available to all researchers, thereby ensuring that ongoing value can be extracted from this important genomic and proteomic resource created in the project.

Other recommendations arising from this project are:

- That the list of potential vaccine candidates identified in this project be further assessed to determine their role in maggot survival through validation in the fly.
- That AWI consider additional strategies for example Wolbachia to help control the sheep blowfly.

1. Introduction/Hypothesis

Despite efforts to control flystrike, including chemical (insecticides), genetic (selective breeding of sheep and fly transformation), physical (mulesing and crutching) and biological (exploiting existing entomopathogens) methods (reviewed by Sandeman et al., 2014; Anstead et al., 2016; Anstead et al., 2017), there are still significant issues with current control options that must be addressed to enable growers to economically and effectively control flystrike while also responding to the concerns of animal welfare organisations.

To design new control strategies (e.g., vaccines) and to enhance current practices (e.g., insecticides), there is a pressing need to fill some of the fundamental knowledge gaps in our understanding of blowfly population biology. This study addressed these gaps using recent advances in next-generation sequencing and high-density variant detection methods to conduct a detailed analysis of blowfly samples collected from across Australia, making a major contribution towards our knowledge of blowfly population structure. We also conducted studies on maggot proteins and investigated sheep responses involved in the early stages of flystrike that will aid in the search for novel control options such as vaccines. This was achieved through both our analysis of the proteins excreted during the early stages of flystrike and through our collaboration on relevant elements of this work with an AWI/CSIRO project (ON-00619) trialing several proteins as antigens for a vaccine against flystrike.

2. Literature Review

The population biology and parasitism of the Australian sheep blowfly, *Lucilia cuprina dorsalis.*

L. cuprina dorsalis (*L. c. dorsalis*) is a major ectoparasitic pest of sheep associated with 90% of skin myiasis (flystrike) in sheep. The damage from this pest and associated costs to the Australian wool growing industry have risen from the estimated ~$170M annually in 2015 (Lane, Jubb, Shephard, Webb-Ware, & Fordyce, 2015) to annual costs of $324M to the producers (Shephard, Webb-Ware, Blomfield and Niethe, 2022). Many blowfly species feed on carrion and garbage; however, the Australian sheep blowfly (*L. c. dorsalis*) has evolved as a significant parasite of sheep, with some breeds more susceptible to flystrike, particularly Merino but also Merino crossbred sheep (Sandeman et al., 2014). This blowfly inflicts wounds on its sheep host, developing through its larval stages by feeding on wound secretions prior to dropping to the ground and burrowing into the soil for the pupal stage of growth until adults emerge to restart this cycle. This blowfly also has the capacity to overwinter in the soil (Wardhaugh, Read, & Neave, 1984).

The introduction of *L. c. dorsalis* to Australia was thought to have occurred via agricultural shipments travelling enroute through South Africa or India at multiple times in the 1800's. Incursions have been proposed to have occurred in both Northwest Australia and Southeast Queensland (Norris, 1990; Waterhouse & Paramonov, 1950). The first flystrike was recorded around 1883 in Australia and hence the fly was presumably present prior to this time. There has also been some debate about the origins of this fly's propensity to cause flystrike on sheep. Norris (1990) postulated the increased prevalence of flystrike was potentially associated with the introduction of the American Vermont genetics into Australian Merino flocks which possessed traits including a heavier fleece and extensively wrinkled skin which contributed to a higher susceptibility to flystrike (Norris, 1990). Interestingly, *L. c dorsalis* had not been reported in Tasmania before the 1950's (identified by (Ryan, 1954), while by 1937, *L. c. dorsalis* was reported in all mainland states (Mackerras & Fuller, 1937), which again supports the notion that the species was introduced rather than native to Australia.

Other *Lucilia* species are also present in Australia, including a closely related species, *Lucilia sericata*. However, Mackerras and Mackerras reported that *L. c. dorsalis* is the primary initiator of flystrike and has been shown to be significantly more selective for laying eggs on sheep over *L. sericata* (Wardhaugh, Mahon, & Bedo, 2001). Evidence for this comes from an examination of blowflies recovered from strike sites on sheep which found that >50% of strikes which involved *L. cuprina* consisted of this species alone, while in the case of *L. sericata*, it was found to be feeding alongside other fly species 96% of the time (Waterhouse & Paramonov, 1950). Further evidence against *L. sericata* as

a major initiator of flystrike also came from studies on oviposition preferences, where *L. sericata* was rarely laid eggs on sheep unless the sheep were primed by the presence of *L. c. dorsalis* eggs (Wardhaugh, Read, & Neave, 1984).

There are two subspecies of *L. cuprina: L. cuprina cuprina* (Wiedemann) (*L.c.c*) and *L. cuprina dorsalis* R-D (*L.c.d*). These two subspecies are found globally in warmer sub-equatorial regions; *L.c.c* in Asia, the Pacific and the Americas and *L.c.d* in Africa, subcontinental Asia and Australia (Stevens and Wall 1996, Stevens, Wall et al. 2002, Wallman, Leys et al. 2005, Wells, Wall et al. 2007, Harvey, Gaudieri et al. 2008, Tourle, Downie et al. 2009, DeBry, Timm et al. 2010, Nelson, Lambkin et al., 2012). Interestingly, there is prior evidence that these two subspecies can interbreed (Norris 1990). *L. sericata,* has a cosmopolitan distribution across temperate countries (i.e., UK, Europe, Australia, New Zealand, Africa, Asia, Pacific and the Americas) and has been used as a forensic tool and as a medical therapy (Stevens & Wall, 1996). *L. sericata* has not been reported as a major pest in Australia; however, it does co-exist in regions with *L. cuprina* and hybrids have also been produced from these species (Stevens and Wall 1996, Stevens, Wall et al., 2002). The potential that hybridization is possible and that hybrid zones of these blowfly species could exist highlights a need to gain further insights into the population biology of *L. c. dorsalis* in Australia, both from an evolutionary perspective and for practical applications to pest management programs.

Prior studies examining blowfly population structure have relied upon the use of allozymes, Random Amplified Polymorphic DNA (RAPD) typing and mitochondrial sequences (Gleeson & Heath, 2010; Gleeson & Sarre, 1997; Stevens and Wall 1995;1997). These studies have helped address the relationships between several closely related *Lucilia* spp. However, there is still only a limited understanding of how much gene flow and migration occurs in Australia and whether blowflies should be considered as a single panmictic population or whether there is a distinct population structure. Studies in New Zealand, where *L. c. dorsalis* was identified in 1988 predicted that the species arrived in the late 1970's (Holloway, 1991). Researchers found that it did not disperse well, particularly when hosts were present (Heath, Bishop, Cole, & Dymock, 1991), that there was only moderate gene flow and that there was little isolation by distance (Gleeson & Heath, 2010). In Australia females are also reported to be found in higher ratios around sheep campsites, and their distribution is quite concentrated around sheep sites, with much lower densities in between properties and paddocks not containing sheep (Wardhaugh et al., 1984). At a local level, the movement of flies between areas based on release and recapture has been previously reported as quite limited. Those migrating individuals recaptured travelled distances ranging up to 4.7 miles (~7.5km) over <30 hours (Gilmour, Waterhouse et al. 1946). At the time these studies were performed, the methods utilised were quite advanced, however modern sequencing and genomics technologies now offer the potential to achieve greater sequence variant resolution for studying population structure.

In this study reported here we sought to utilise these next generation sequencing technologies to characterise the population structure of the Australian sheep blowfly, addressing this clearly identified gap in our understanding of blowfly biology. We conducted a nation-wide survey of blowfly samples and then employed pooled whole genome sequencing and modified ddRADseq approaches using DNA extracted from these samples to examine the genomic variation and frequencies of high-density molecular markers. Bioinformatic analyses allowed us to identify regions that are specific to distinct blowfly populations and to determine the population structure present for *L. cuprina dorsalis* in Australia. We have also been able to examine the variation present in blowflies across Australia to show levels of migration between regions. This information has been useful to help inform our colleagues at the CSIRO, through providing data on variation in antigens of interest in their research and has significant value to evaluating the potential of area-wide control strategies (such as Sterile Insect Technique).

Understanding of the host-parasite interaction is key to developing a vaccine against flystrike.

There are multiple scenarios that can lead to flies parasitising sheep. In one case, the sheep may have a pre-existing wound or some other form of skin damage that flies may lay eggs directly on and does not require the formation of a wound by the parasites themselves. In other cases, however, flies identify suitable sites to lay eggs on intact skin that are associated with predisposing factors such as dags, urine staining, scouring and bacterial infections such as fleece

rot (Kotze & James, 2022). Following hatching, maggots can feed on detritus and proteins in the fleece and migrate towards the skin of sheep. First instar maggots can also abrade the skin both mechanically with their serrated mouth hooks while also excreting proteases and other factors that enable them to stimulate the flow of nutrients from the wound (Sandeman, Collins, & Carnegie, 1987). In response to the presence of the larvae, sheep were found to produce antibodies that could recognise maggot antigens during flystrike, but the presence of an antibody response did not lead to a protective immune response (Eisemann et al., 1990; Sandeman, Bowles, Stacey, & Carnegie, 1986; Sandeman, Dowse, & Carnegie, 1985). Interestingly previously struck sheep were not found to have higher antibody titres than sheep following a lesser exposure (Elliott, Nagy, Takacs, Ben-Neriah, & Givol, 1980) nor were significant differences in lymphocyte subpopulations in sheep during secondary infections with the larvae found (Bowles, Grey, & Brandon, 1992).

Significant efforts to identify potential vaccine antigens in the 1980s and 1990s identified several proteins that could induce antibody responses in sheep that could inhibit maggot growth, but not necessarily lead to the death of, maggots. The majority of the proteins were either from secretion products or from gut proteins (Casu et al., 1997; Eisemann & Binnington, 1994; Johnston, Eisemann, Donaldson, Pearson, & Vuocolo, 1992; O'donnell, Green, Connell, & Hopkins, 1981; R. Tellam & Eisemann, 1998; R. Tellam et al., 2003; R. L. Tellam et al., 2001). Despite this research, no vaccine candidates were identified that led to a commercially viable vaccine. One of the significant lessons to take away from these early vaccination studies was that without a more detailed understanding of the host-parasite interactions, it will continue to be very difficult to identify suitable candidate antigens and to predict which of those candidates may elicit suitable protective responses. A greater knowledge of this area would be a significant benefit for future efforts to identify suitable antigens and the types of immune response in the sheep that may offer higher levels of protection.

However, some important findings arose from this earlier work that were useful in informing future studies. These findings note that native antigens produced better protective responses than recombinant antigens, suggesting that accurate post-translational modifications were important for eliciting responses (Bowles, 2001). The production of recombinant proteins may be the best solution for the longer term to producing antigens at scale, but an important consideration in the development of such a vaccine are the productions costs which need to be at a competitive price point in order to produce a commercially viable end-product. An additional confounding factor was identified that related to the presence of immunomodulatory products produced by the larvae that could potentially impact the response of the sheep immune system to infection (Kerlin & Hughes, 1992). One of these potential immunomodulatory molecules was found to be a 56Kd, as yet unidentified protein, that could inhibit lymphocyte proliferation (Elkington et al., 2009). Another interesting observation was a report indicating that immune responses which appeared to be most directly associated with a protective host response related to a cellular rather than a humoral component (Bowles et al., 1996). Clearly further study was warranted in order to more fully address the lack of knowledge around the proteins secreted by *L. cuprina* maggots to establish the wound and the subsequent type of responses that is instigated by sheep right at the time that a larva commences to initiate a wound. We aimed to address both of these areas through the use of bioinformatics to identify blowfly and sheep proteins present at flystrike wounds. These findings should aid efforts towards identifying the nature of the sheep immune response during wound initiation, and also in the identification of suitable antigens for the development of vaccines to protect sheep against flystrike.

3. Project Objectives

Objective 1. *L. cuprina* population sampling across Australia during the 2018/19, 2019/20 and 2020/21 flystrike seasons

Objective 1.

- Recruit growers and researchers to assist in the collection process. The collection locations will include sites across NSW, VIC, QLD, WA, TAS and SA. In-depth sampling will occur in Jan-April of 2019 and across the entire 2019/20 season. Targeted sampling in 2020/21 season will be undertaken based on earlier findings.
- Plan and conduct on-site sampling in specific rural and urban areas.
- Assemble and distribute sampling kits, with samples returned by April.
- Extract and quantify genomic DNA, prepare samples for variation analysis and sequence pooled sample libraries.
- Mapping of sequencing reads to genome.
- Analysis of genetic variation and population structure.
- Use multi-season data to build and refine model of gene flow.
- Examine collected samples for resistance allele frequencies.
- Produce information sheets to educate growers about the blowfly population structure in their region and implications for their pest management strategies.

Objective 2. *In vivo* sheep implant trial to characterise blowfly/sheep biological pathways involved in myiasis

Objective 2

- Submit and obtain ethics approval for sheep trials.
- Conduct sheep trials, collecting larval and sheep samples across the establishment phase of myiasis.
- Prepare samples for proteomics analysis and run raw sample analysis at the Mass Spectrometry and Proteomics Facility at Bio21 Institute.
- Analyse proteomics results.
- Quantify the levels/timing of expression of CSIRO candidate proteins.
- Compare blowfly proteomics with the blowfly transcriptome.
- Determine timing and profile of sheep proteome (response) during implantation.

4. Success in Achieving Objectives

The project has successfully completed the aims of Objective 1 through collection and subsequent genomic analysis of *Lucilia cuprina* samples from across the country over the three collection periods (2019, 2020/21 and 2021/22). Significant genetic variation in the flies was uncovered using the individual DArTSeq and Pool-seq approaches and using these data we were able to discriminate between flies from three population regions of Australia and identify gene flow patterns within these populations. A single large east coast population comprised flies from South Australia through Victoria, New South Wales and into Queensland. The other two populations were flies from Tasmania and flies from Western Australia, respectively. Hence, we discovered that there are separate populations of blowflies present in these geographically distinct regions. We also found that there was no evidence of genetic changes that would be associated with target site resistance to imidacloprid or spinosad, two blowfly insecticides, and are confident our approach was valid given that we identified resistance alleles that have previously arisen to older chemicals, dieldrin, malathion and diazinon.

We have also made significant progress in the understanding of the interaction between blowfly maggots and sheep during the early stage of wound initiation through our sheep implant trial experiments and subsequent proteomic analysis. We successfully identified proteins that are being excreted by maggots and proteins that are found at the sheep wound site, providing detailed insights into this complex biological process. The analysis revealed the presence of several proteins that are of interest to CSIRO and their program to develop a vaccine against flystrike. We also compared highly expressed genes from a previously generated blowfly transcriptome Perry and Bowles (unpublished) to those proteins identified in the current study using proteomics. This approach has validated proteins that we had previously predicted to be secreted during the early stages of maggot development as well as identifying a range of additional proteins that had not been identified using gene expression data but were found to be present and at relatively high levels during maggot development. These studies are assisting us in our efforts to identify further candidate proteins that may contribute towards the development of therapeutics, such as vaccines, that could assist in protecting sheep against flystrike.

5. Methodology

Methods for Objective 1

Blowfly sample collections

Fly material was sourced from 86 sites across Australia and consisted of either adults collected in Lucitraps™ and preserved in a salt solution (RNAlater®) or as maggots from struck sheep (either live and reared to adults in an insectary prior to preservation, or as larvae preserved in RNAlater®). Identified using morphology and based on the three years of sample collections, there were 3250 individual flies (2915 *L. cuprina* and 335 *L. sericata*) from 160 sample collections across 86 sheep properties throughout VIC, NSW, QLD, SA, WA and TAS (Table 1, Figure 1). Adult heads were removed, and DNA extracted as per DArTseq (protocol), or genomic DNA from whole larvae was extracted using CTAB/phenol chloroform and DNA again provided to DArTseq.

Table 1. Number of *L. cuprina* and *L. sericata* flies collected for three years of collection.

Year	Sample collections	Number of *L. cuprina* flies	Number of *L. sericata* flies
2018/2019	30	413	59
2019/2020	81	1235	256
2020/2021	49 (10 adult flies + 39 pupal samples)	1267	20
Total	160	2915	335

In addition to the samples of adults and pupae collected in Table 1, a total of 1142 larvae [(299 larvae (2019/2020) and 843 larvae (2020/2021)] were also collected from 36 locations spread across 5 states (QLD, VIC, NSW, SA and WA) (see Table 2). These samples were often sent from locations where we could not send traps to, allowing us to cover a greater number of regions (particularly helpful during the travel restrictions encountered during COVID lockdowns) and were generally collected directly from struck sheep.

Table 2. Collection sites of larvae collected from 2018/2019-2020/2021.

Year	Sample collection sites	*Lucilia* larvae
2018/2019	0	0
2019/2020	12	299
2020/2021	24	843
Total	36	1142

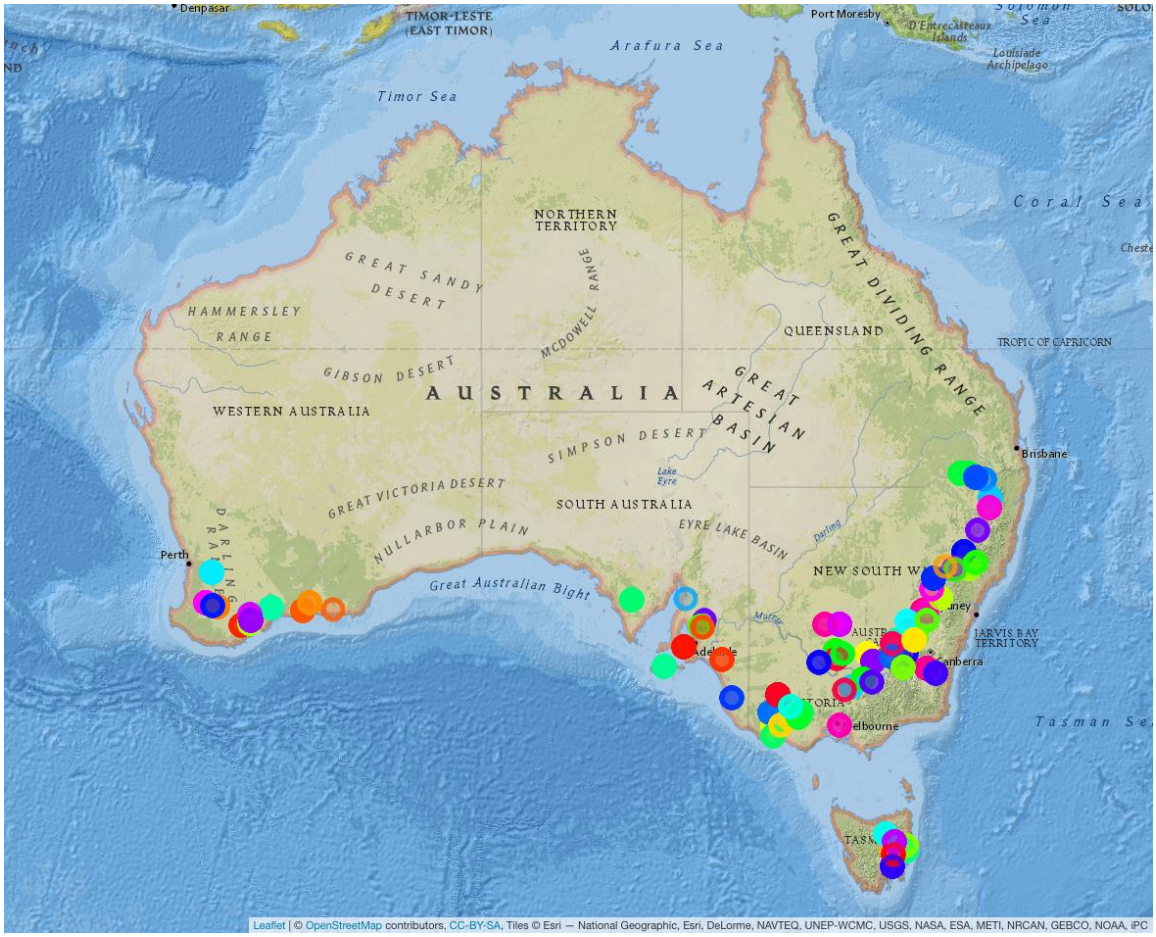


Figure 1. Map showing total *L. cuprina* collection sites for three collection years (2018/2019, 2019/2020 and 2020/2021). These sample sites were used for the multi-year population structure analyses. Different colours indicate the sites from where the blowfly samples were collected.

DArTseq analysis

Samples were again identified and prepared for analysis using DArTseq, which provided information about the variation present in fly DNA at thousands of loci across the genome. DArTseq produced a reduced library and sequenced the samples on an Illumina Hiseq2500 system (https://www.diversityarrays.com/). The technology was optimized for the blowfly genome using combinations of enzymes to select the most appropriate complexity reduction method. After filtering out loci with reproducibility lower than 100% and missing data lower than 15%, the final data set of 26,277 loci was used for the downstream analysis. Input data provided by DArTseq contained alleles coded as '0' (homozygosity with reference allele), '1' (homozygosity with alternative allele) and '2' (heterozygote). The processed marker data were reformatted into appropriate file types for downstream analyses using dartR package (Gruber, Unmack, Berry, & Georges, 2018).

A collective analysis of *L. cuprina* genetic data for the last three years 2018/2019, 2019/2020 and 2020/2021 provided additional collection sites and flies for the analysis allowing for a better resolution of blowfly population structure across Australia. A total of 26,277 polymorphic loci were studied in 2034 individual flies and larvae from 86 *L. cuprina* populations spread across QLD, VIC, SA, NSW, WA and TAS (Figure 2).

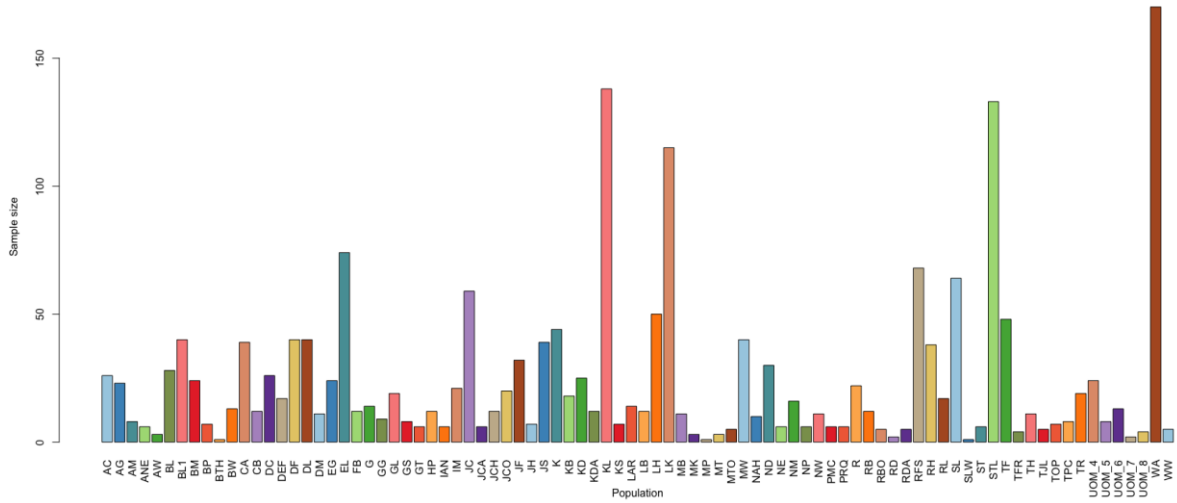


Figure 2. Number of *L. cuprina* individuals per population. Total: 86 populations.

Pool-Sequencing (Pool-seq)

Following DArTseq genetic variation analyses, genomic DNA of 17 *L. cuprina* flies per population was pooled from 61 sites across different states in Australia (Table 3, Figure 3). Raw sequence read quality generated from sequencing was evaluated using the FastqQC program v. 0.11.9 (Andrews, 2010). The software Trimmomatic v.0.39 was used for quality filtering (i.e., removal of adapters, contaminants, low quality (Phred scores < 30) and short (< 50 bp) sequencing reads) (Bolger, Lohse, & Usadel, 2014). The high-quality sequencing reads were mapped back to the reference genome of *L. cuprina* (Freeze_2_UOM, Bioproject number: PRJNA419080) using the MEM algorithm of BWA (parameters: mem -t 4 -k 32 -M) (Li & Durbin, 2009). The SAM output files were converted into sorted BAM files using SAMtools (Li et al., 2009) and duplicate reads were removed using PICARD (Rohland & Reich, 2012). The BAM files for all pools were then merged using the mpileup command of SAMtools with default parameters. Variant calling was done using SAMtools with the following parameter: mpileup -m 2 -F 0.002 -d 1000'. To reduce the error rate in SNP detection, results were filtered with the following criteria: The number of support reads for each SNP should be >4 and the mapping quality (MQ) of each SNP should be >20 (Li et al., 2009). The statistics from sequencing data for *L. cuprina* are provided in Appendix 1.

Table 3. Number of *L. cuprina* field collected samples that were whole genome re-sequenced.

State	Pooled *L. cuprina* samples
QLD	8
NSW	16
VIC	17
TAS	2
SA	6
WA	11
NT	1
Total	61

Figure 3. Map of collection sites for *L. cuprina* samples used for pool sequencing.

Genome-wide analyses of genetic differentiation

Six pools were selected based on their genetic values (from DArTseq distance matrix) from WA, VIC and NSW respectively, and merged to form each state pool. A total of 4 pools from SA, 3 pools from QLD and 2 pools from TAS were merged for each state (Figure 4). The regions of the genome that differed between the sequenced pools were identified using create-genewise-sync.pl and fst-test.pl (parameters: --min-count 2 --min-coverage 4 --max-coverage 2% --pool-size 102:102:34:102:51:68) using the Popoolation2 program (Kofler, Pandey, & Schlötterer, 2011). Pairwise comparisons were made between the merged pools from WA, NSW, VIC, TAS, SA and QLD. The genome-wide mean fixation index was calculated for all the pairwise comparisons by taking the mean of all the FST values obtained for individual SNPs. A conservative cut-off approach was taken (FST > genome-wide mean FST$\pm$1SD) to determine whether the high FST value for an individual gene is likely to be simply "noise" or indicative of a region in the genome that is, or has relatively recently undergone, a period of selection, and was linked with at least one other high FST gene.

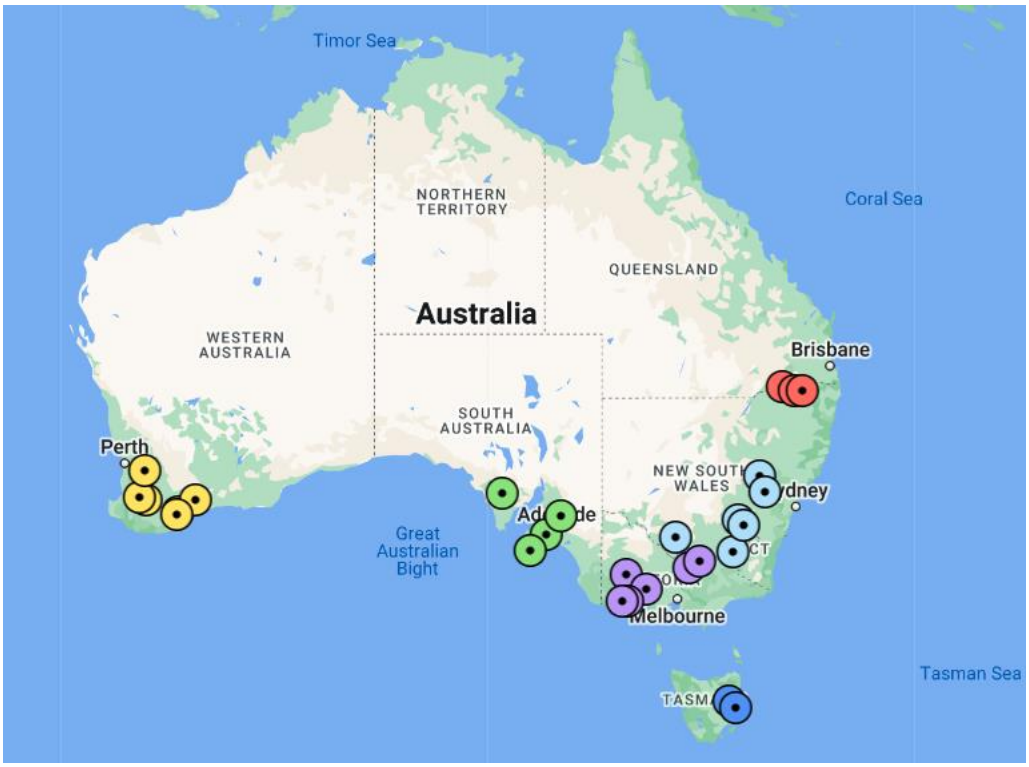


Figure 4. Map of the pools of *L. cuprina* for which the data was merged for each state for FST analysis. The red dots represent the merged QLD pool, green dots represent the merged SA pool, yellow dots represent the merged WA pool, purple dots represent the merged VIC pool, light blue dots represent the merged NSW pool and dark blue dots represent the merged TAS pool.

Alignments for insecticide resistance

The protein sequences for each *L. cuprina* insecticide resistant gene were extracted from the Pool-seq data and aligned for all the blowfly field-collected and the lab strain (Freeze_2_UOM, Bioproject number: PRJNA419080) using Muscle v.3.8.425 (Edgar, 2004) in the Geneious Prime v.2019.2.3 software (Kearse et al., 2012). The alignments for each gene were checked for the previously reported insecticide resistance and susceptible amino acid changes.

Allele frequency

The detailed statistics about the major and minor alleles for all SNPs in the given populations were calculated using snp-frequency-diff.pl script from Popoolation2 (Kofler et al., 2011). The data was used to extract the frequencies of insecticide resistance and susceptible alleles.

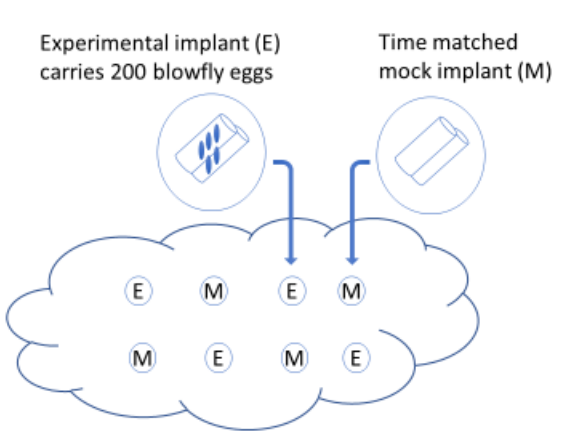
Methods for Objective 2

Implantation of blowfly eggs on sheep.

An ethics application was submitted that detailed the nature of the experiment to be conducted on sheep. A total of 14 sheep (7 from the breech-strike resistant flock and 7 from the non-selected flock) were used in this study. These sheep were sourced from Armidale, New England Merino Lifetime Productivity Project (Dr Jen Smith, CSIRO Chiswick campus). The experiment took place at the Animal House at the University of Melbourne (UOM) in Parkville.

The procedure used for obtaining larval samples was designed to represent conditions present on a live sheep that will permit blowfly eggs to hatch and the larvae to initiate a wound. Four experimental implant sites (eggs on dental plugs) and four mock implant sites (dental plugs only) per sheep were set up to enable a time course experiment to be undertaken (see figure 5 below).

Figure 5.



Implantation Schedule

- 1pm (1st day) Paired sites (E and M) implanted
- 3pm (1st day) sheep inspected
- 7pm (1st day) sheep inspected
- 7am (2nd day) sheep inspected
  Paired sites (E and M) collected at 3-hour intervals (4 collections);
- 10am (2nd day) collection 1.
- 1pm (2nd day) collection 2.
- 4pm (2nd day) collection 3.
- 7pm (2nd day) collection 4.

After collection of larvae at 7pm

- Serum sample taken
- Sheep euthansed
- Biopsies from all 8 sites

Schematic of a top view of sheep's back with experimental design.

- Experimental implant sites (E) alternated with mock implant sites (M).
- An adjacent pair of E and M will be collected at each timepoint (randomized selection of paired sites).
- On removal, the site will be inspected for larvae, the wool at the site sheared and the site again inspected to remove any larvae present.
- After larvae are collected at 7pm a serum sample will be taken. Sheep will be euthanised and then biopsies taken for all E and M sites.

In short, the process involved selecting an area on the back of the sheep where wool is present and identifying an area approximately 5cm in diameter. The wooled area was thoroughly wetted with water and 200 blowfly eggs placed onto two moist dental plugs and the damp section of the wool parted to allow positioning of the implant as close as possible to the skin surface. The parted wool is then gathered around to cover the dental plugs and an elastic band is used to secure wool over the implant site to stop the dental plugs from falling off the sheep. Under these conditions the eggs would be expected to hatch within 20-24 hours.

Sample collection

Samples were collected from sheep implant sites at different stages of wound initiation and included whole maggot recovery, maggot excretions/secretions, wound and skin exudate and biopsies of both wound- and mock-implanted-skin sites (refer Figure 5). For comparison with these implanted eggs, two different controls were used. The first control involved placing the cotton plugs used for the implants onto sheep without eggs (skin exudate and a biopsy of the site were sampled) and the other control involved using eggs that were undergoing development in a petri dish with only the cotton plugs present. For these samples, eggs were reared on the same wet cotton plugs as implanted eggs; however, these were placed in a petri dish and reared at 27°C with samples collected at the same timepoints as the implant assay maggots (whole maggots, maggot excretions/secretions were collected). The protein samples from both maggots and sheep were collected in a PBS/protease inhibitor solution and snap frozen in liquid nitrogen prior to storage and subsequent analysis.

Sample digestion and preparation for peptide identification

Prior to trypsin digestion, samples were quantified using a Pierce BSA assay to determine protein concentration and then processed using a single pot digestion modified from Hughes et al (2019). Samples were freeze dried and resuspended in 15ul mass spec loading buffer prior to submission for peptide sequencing. Peptide sequencing results were processed using MaxQuant (Cox and Mann, 2008) using the Sheep (Oar.) and blowfly reference proteomes (Freeze_2_UOM, Bioproject number: PRJNA419080) for identification of peptides present. Statistical analysis of the peptide intensities was performed using Perseus (Tyanova et al, 2016).

Histology

Skin samples were processed for either paraffin Hematoxylin/Eosin or frozen for immunohistological analysis. In short, for immunohistology skin sections were embedded in Optimal Cutting Temperature Compound (Tissue-Tek, Miles) and frozen in liquid nitrogen. Skin sections (5/microns) were placed on glass slides before being immersed in absolute ethanol containing 1% hydrogen peroxide for fixation and to remove any endogenous peroxide. The sections were then air-dried before being subjected to indirect immunoperoxidase staining according to the method described by (Meeusen, Gorrell, & Brandon, 1988). Slides were lightly counterstained with haematoxylin. The skin sections were stained for CD4+, CD8+, Gamma-delta+ and T19+ T cells as well as B cells and CD1+ Langerhans cells.

6. Results

Population Genomics

High-density molecular markers were used to achieve a greater resolution of the population structure of the Australian sheep blowfly in this study. Broad coverage of natural populations was achieved from collection of blowfly samples from 86 sites across Australia during the 2019, 2019/20 and 2020/21 blowfly seasons. Our results clearly demonstrate that the blowfly population samples belong in three genetically distinct clusters. The genetic distance between populations demonstrates a high level of divergence between interstate populations of WA and TAS compared to all other populations. The individuals/populations were partitioned into three distinct clusters (coloured by the populations they originate from), revealing the presence of distinct genetic differentiation between the flies in these three clusters (Figure 6). The VIC, SA, QLD and NSW populations aggregated together as a mixed cluster (the Eastern Australia cluster) whereas the Tasmanian blowfly populations aggregated into a distinct cluster. The Western Australian populations formed another distinct cluster in the DAPC plot. There is dispersal, gene flow and admixture between the populations within each of the clusters.

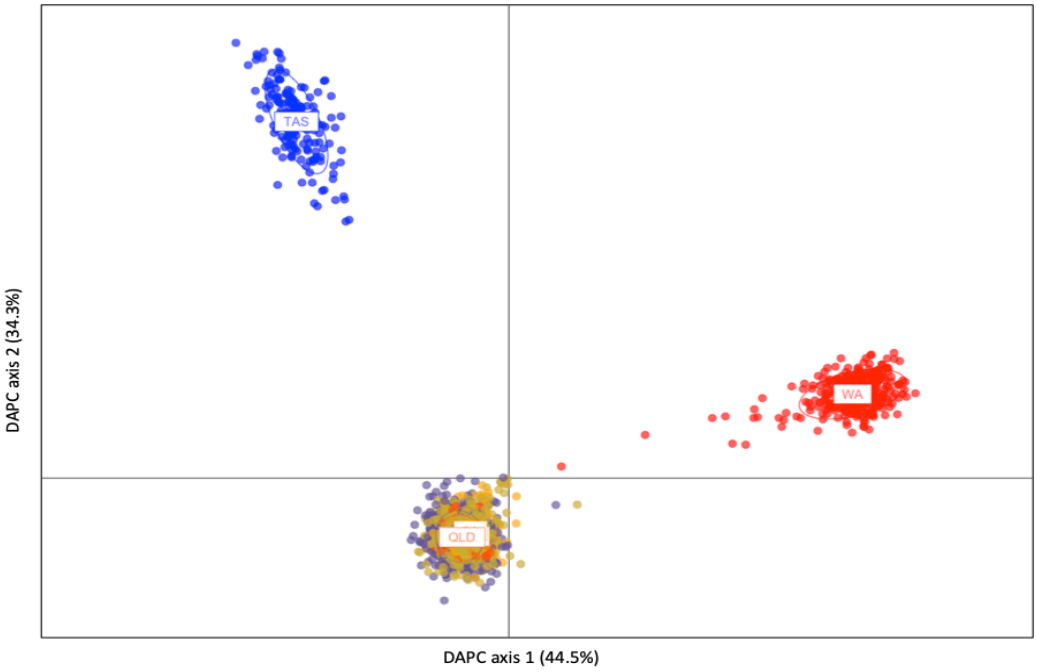


Figure 6. Discriminant analysis of principal components (DAPC) plot. Populations are clearly divided into three distinct clusters with dispersal, gene flow and admixture between the populations in each cluster. DAPC assigned individuals to groups based on extracting genetic information from allele frequency data and outputs a visual assessment of between-population differentiation. Each dot represents one individual. The populations from Eastern Australia (VIC, NSW, QLD and SA) cluster together. The populations from Western Australia and Tasmania form two genetically distinct clusters.

The WA and TAS fly populations are clearly separated from the Eastern Australian population cluster. There is evidence that the fly populations from WA migrate within the state but due to the geographical distance do not interbreed with the Eastern Australian fly populations. Similarly, the Tasmanian populations are genetically different to the Eastern and Western Australian populations. Geographic distance plays an important role in keeping the WA and TAS

populations isolated from Eastern Australian populations, while there is evident gene flow between populations from QLD, SA, NSW and VIC. Therefore, for all Australian blowfly populations, proximity plays an important factor for these populations to migrate and breed with each other.

Genome-wide analyses of genetic differentiation (2021/2022)

The pairwise FST value provides an indication of whether a specific region of the genome is the same or different for the two populations being compared. The value can be between 0 and 1, with a value of 0 indicating genetically identical and a value of 1 indicating that they are completely different. For this analysis, the regions in the genome that demonstrated significant genetic differentiation between the pairwise comparisons were defined as the genes that had FST values >1 standard deviations from the genome-wide mean FST and which were linked with at least 1 other gene with a high FST value (high FST values means that the region is different between the populations being compared). This approach allowed us to determine whether the high FST value for a gene is likely to be "noise" or a significant signal indicative of a genomic region experiencing selection. The genes present in these genomic regions were further analysed. Mean fixation index was calculated for all pairwise comparisons and cut-off values (Table 4) were used to shortlist the candidate genes under selective pressure (Figure 7). The mean FST value was highest for the pairwise comparisons between WA vs TAS (0.05996336) which means they are highly genetically different from each other (Table 5). The mean FST value for AS field-collected population from Northern Territory was also examined in a pairwise comparison to one of the NSW field-collected population (UOM56) which is 0.1559. The mean FST value for the pairwise comparison of AS population with one of the WA field-collected population (NPSTBP) was 0.1116.

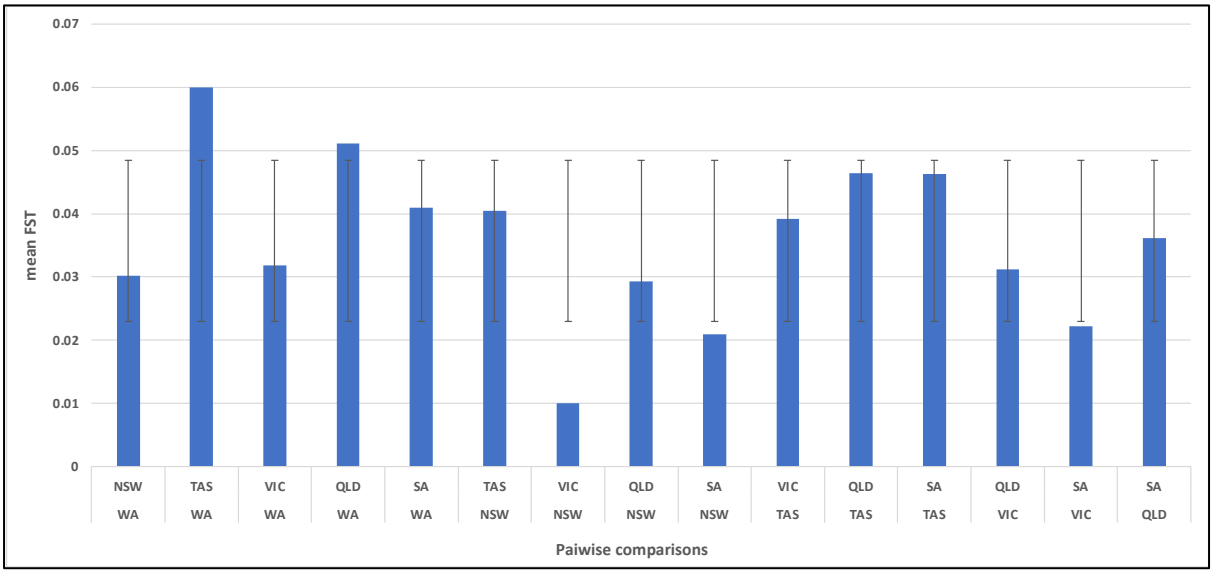


Figure 7. Mean fixation index (FST) values for pairwise pool comparisons.

Table 4. Mean fixation index (FST) ± 1 standard deviation values for pairwise pool comparisons.

Pairwise comparisons		Mean FST	STDEV	Cut off (FST+STDEV)
WA	NSW	0.0301359	0.02413019	0.05426608
WA	TAS	0.05996336	0.04069407	0.10065743
WA	VIC	0.03177519	0.02750488	0.05928007
WA	QLD	0.05110569	0.02928671	0.0803924
WA	SA	0.04092568	0.02851097	0.06943665
NSW	TAS	0.0404045	0.02490074	0.06530524
NSW	VIC	0.01004242	0.00863247	0.01867489
NSW	QLD	0.02926924	0.01522741	0.04449665
NSW	SA	0.02089857	0.01261329	0.03351187
TAS	VIC	0.03912268	0.019946	0.05906868
TAS	QLD	0.04635737	0.02789421	0.07425158
TAS	SA	0.04630238	0.02694055	0.07324293
VIC	QLD	0.03118994	0.01631533	0.04750526
VIC	SA	0.0222212	0.01214512	0.03436633
QLD	SA	0.03615122	0.0203949	0.05654613

Gene Ontology enrichment analysis

The genes in the genomic clusters that met the criteria (see Method section) (Table 5) were selected for the Gene Ontology enrichment analysis for the pairwise comparison between WA vs NSW, WA vs TAS, NSW vs VIC and NSW vs TAS. The Functional Annotation Clustering first performs an analysis of gene enrichment and attempts to group the enriched terms into functional clusters (Huang, Sherman, & Lempicki, 2009; Sherman et al., 2022).

Table 5. The number of genes that met the criterion of candidates for FST analysis.

Pairwise comparisons	Number of genes that met the FST analysis criterion
WA vs NSW	536
WA vs TAS	453
NSW vs VIC	198
NSW vs TAS	463

Of the 536 genes that met the FST analysis criteria in the WA vs NSW pairwise FST analysis, 333 genes grouped into functional clusters in the GO analysis (Figure 8). In the WA vs NSW pool comparison, the genes that were enriched were associated with the following functions: nucleotide binding, GPCR, response to pheromone, ABC transporter, leucine-rich repeat, mitochondrial translation, immunoglobulin-like domain (Figure 8). These are differences that could be expected for *L. cuprina* found in different environments. These genes may be involved with adaptation to the different sensory cues in their respective local environments, and/or may also affect their host- or food-seeking behaviour

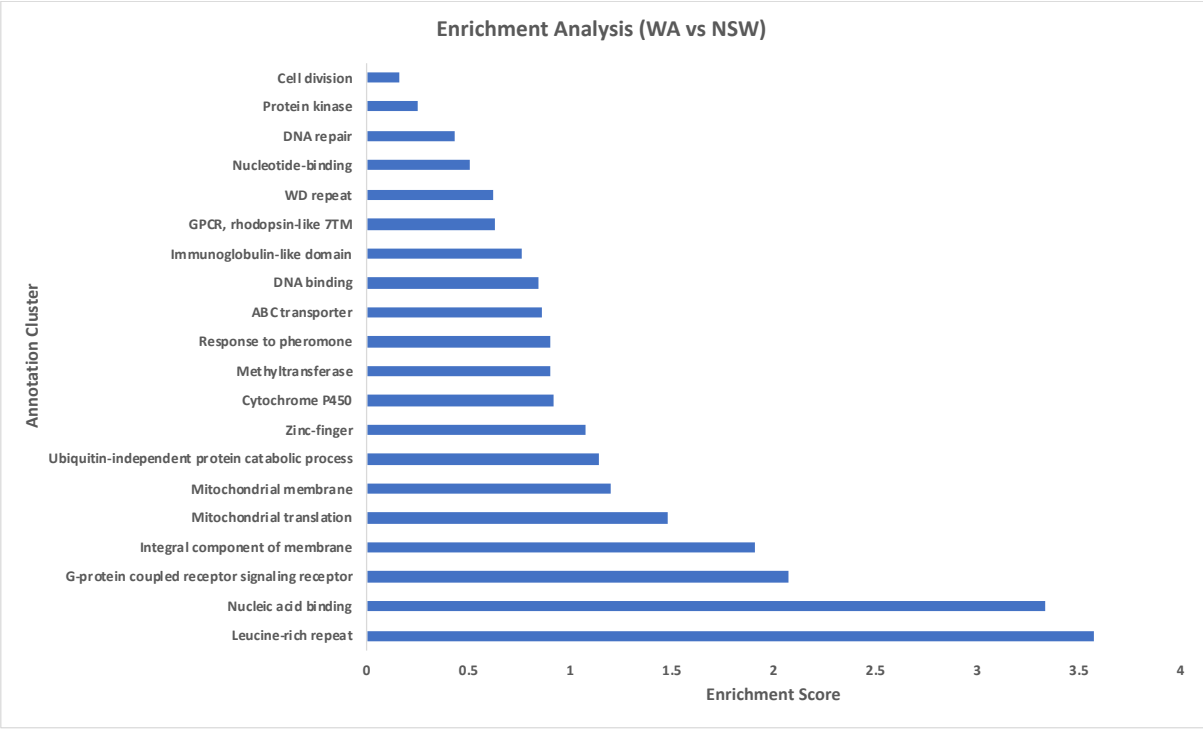


Figure 8. Bar graph of Gene Ontology (GO) enrichment analysis between WA vs NSW pool. The annotation clusters are shown on the Y-axis and their enrichment scores are shown on X-axis.

Of the 453 genes that met the FST analysis criteria in the WA vs TAS pairwise FST analysis, 313 genes grouped into functional clusters in the GO analysis. In the WA vs TAS pool comparison, the genes that enriched in the analysis were associated with DNA binding, olfactory receptor activity, WD40 repeat, GPCR, leucine-rich repeat, and Sodium-ion transport (Figure 9).

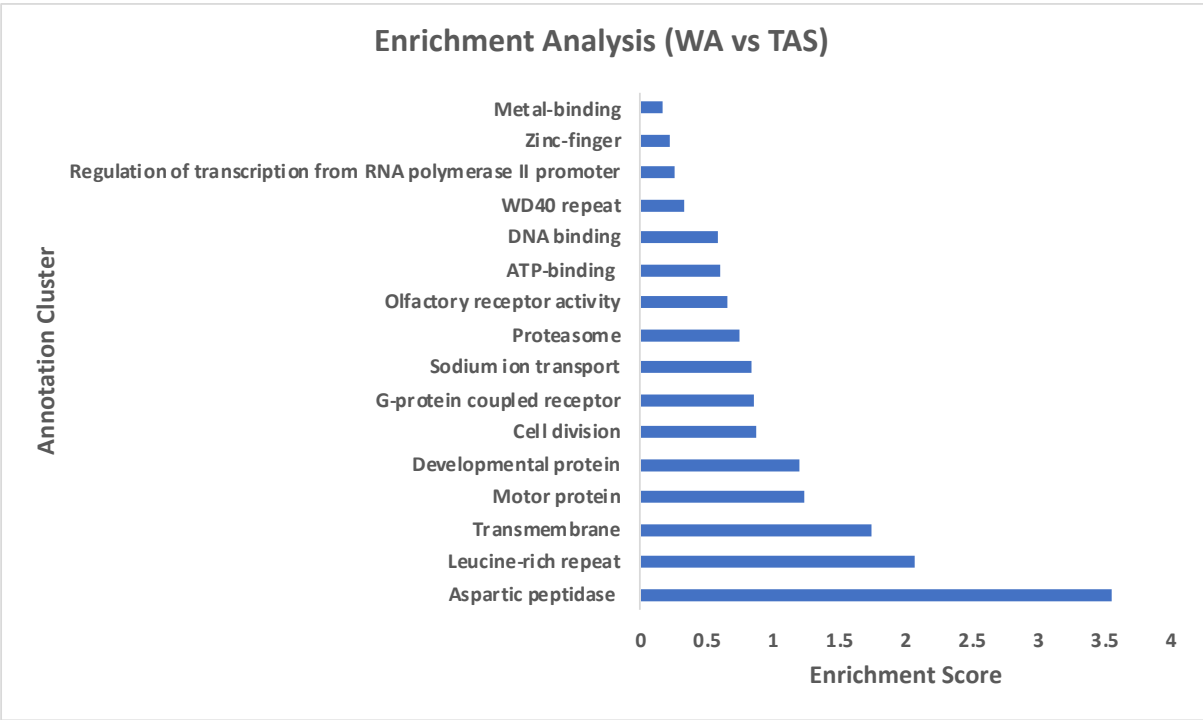


Figure 9. Bar graph of Gene Ontology (GO) enrichment analysis between WA vs TAS pool. The annotation clusters are shown on the Y-axis and their enrichment scores are shown on X-axis.

Of the 463 genes that met the FST analysis criteria in the NSW vs TAS pairwise FST analysis, 321 genes grouped into functional clusters in the GO analysis. In the NSW vs TAS pool comparison, the genes that enriched were associated with signal, mitochondrial, immunoglobulin-like fold, DNA binding, WD40 repeat and Serine/threonine-protein kinase (Figure 10).

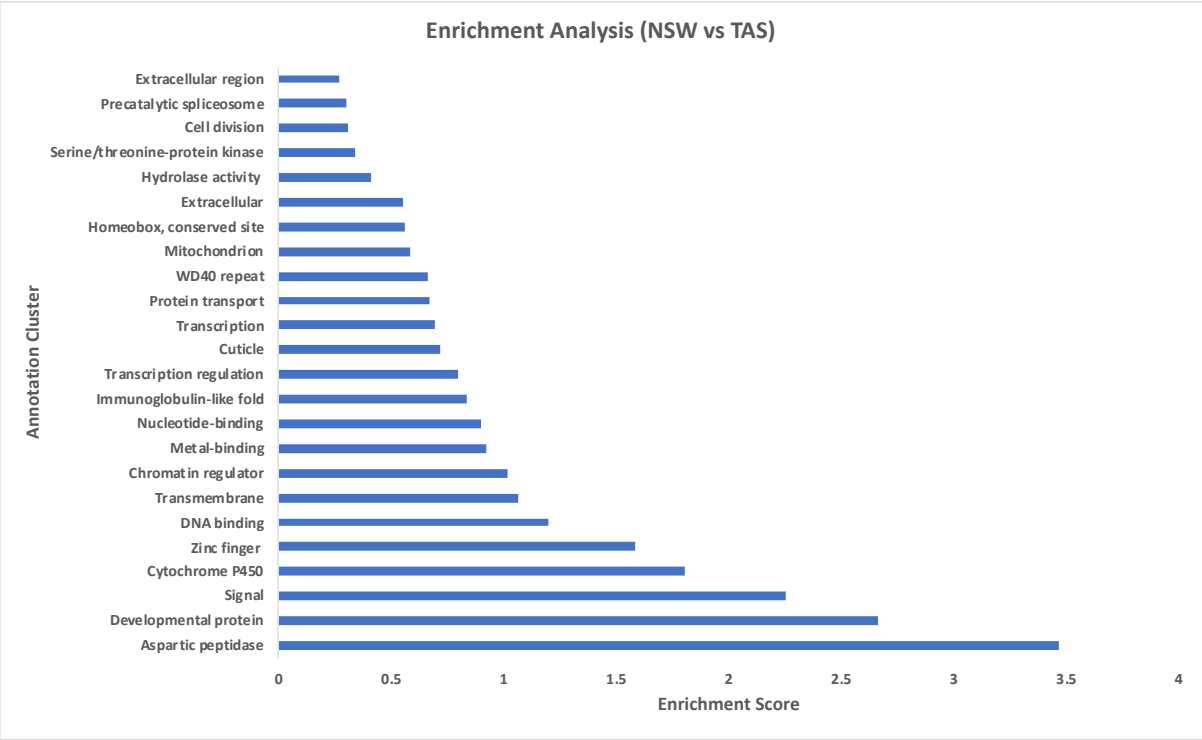


Figure 10. Bar graph of Gene Ontology (GO) enrichment analysis between NSW vs TAS pool. The annotation clusters are shown on the Y-axis and their enrichment scores are shown on X-axis.

Of the 198 genes that met the FST analysis criteria in the NSW vs VIC pairwise FST analysis, 143 genes grouped into functional clusters in the GO analysis. In the NSW vs VIC pool comparison, the genes that enriched were associated with membrane, transcription, zinc finger and Aspartic-type endopeptidase activity (Figure 11). There are four enrichment clusters that were found in all the pairwise pool comparisons. The genes associated with Asparatic-type endopeptidase activity, zinc finger, transcription and membrane were enriched in these clusters

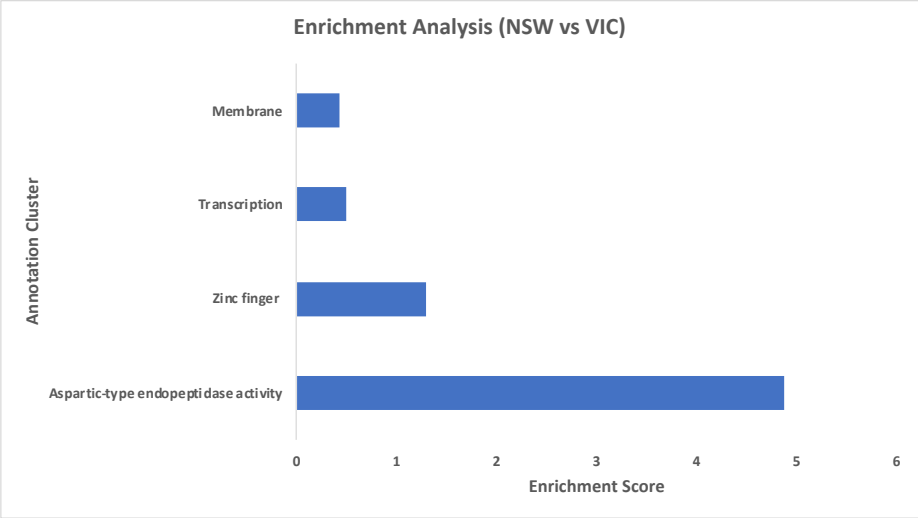


Figure 11. Bar graph of Gene Ontology (GO) enrichment analysis between NSW vs VIC pool. The annotation clusters are shown on the Y-axis and their enrichment scores are shown on X-axis.

The NB flies (CSIRO trial blowfly strain) that were the wild type collected from several paddocks at the CSIRO Chiswick field station were reared in the laboratory for 12 (primed: two feeds of liver) and 13 (unprimed: no liver exposure) generations, respectively. Notably, these flies were genetically distinct to the other flies in our analysis (Appendix 2). This suggests that potentially through the culturing over many generations under laboratory conditions and as a function of the small population size (bottleneck), the population may have undergone genetic drift. This could cause many natural alleles initially present in the founding flies of the population to disappear and drive the NB population towards genetic uniformity over time. This loss of variants may also alter how well these flies would perform under field conditions and their capacity to replicate the likely responses of blowflies in the field. Details from the above analyses during the project have been provided to CSIRO so they have access to all data relevant to their work identifying vaccine candidates (i.e., protein alignments and FST analysis of vaccine candidates) through our collaboration agreement which included regular collaboration meetings to discuss results and through email and phone correspondence to clarify any aspects of the project when required.

Migration rates in WA, TAS and Eastern Australia populations

Migrate-n from MIGRATE-n3.5.1 was used to estimate the migration pattern among the populations (Beerli, 2006). The WA and TAS fly populations are isolated from Eastern Australian populations and there is evidence that the fly populations from WA migrate within the state but due to the geographical distance do not interbreed with the Eastern Australian fly populations. Similarly, the Tasmanian populations are genetically different to the Eastern and Western Australian populations (Figure 5). Geographic distance plays a significant role in keeping the WA and TAS populations isolated from Eastern Australian populations. For the Australian populations the proximity plays an important factor for these populations to migrate and breed with each other. Even though the geographic distance affects the population structure, human-aided dispersal from live sheep transport may also play a role in the migration of flies to distant locations connected by major roads (East & Foreman, 2011). Annual temperature and humidity may also play an important role in the migration of these flies. Our analysis suggests high levels of gene flow in the Eastern Australian populations. The sheep numbers are higher (> 200k sheep) in the regions of our *L. cuprina* collection sites, providing many potential hosts and facilitating movement of flies in these regions. Based on our data, there appears to be bidirectional migration of *L. cuprina* flies and interbreeding in these regions. Estimates for the gene flow rate (Nm) of *L. cuprina* groups in the Eastern Australian region are shown in Figure 12. The filter threshold for the amount of gene flow was set to 0.6. Bidirectional (mixed) gene flow occurred between the populations in the Eastern Australian cluster. The WA and TAS populations form distinct clusters with no gene flow with the interstate state populations.

There is moderate level of gene flow in the clusters of fly subpopulations in WA (Figure 13). The proximity of these sheep properties in WA plays an important factor for these populations to migrate in both directions and breed with each other. The level of gene flow observed in this study may be due to the fact that samples were in close proximity within WA with absence or limited impacts of spatial isolation.

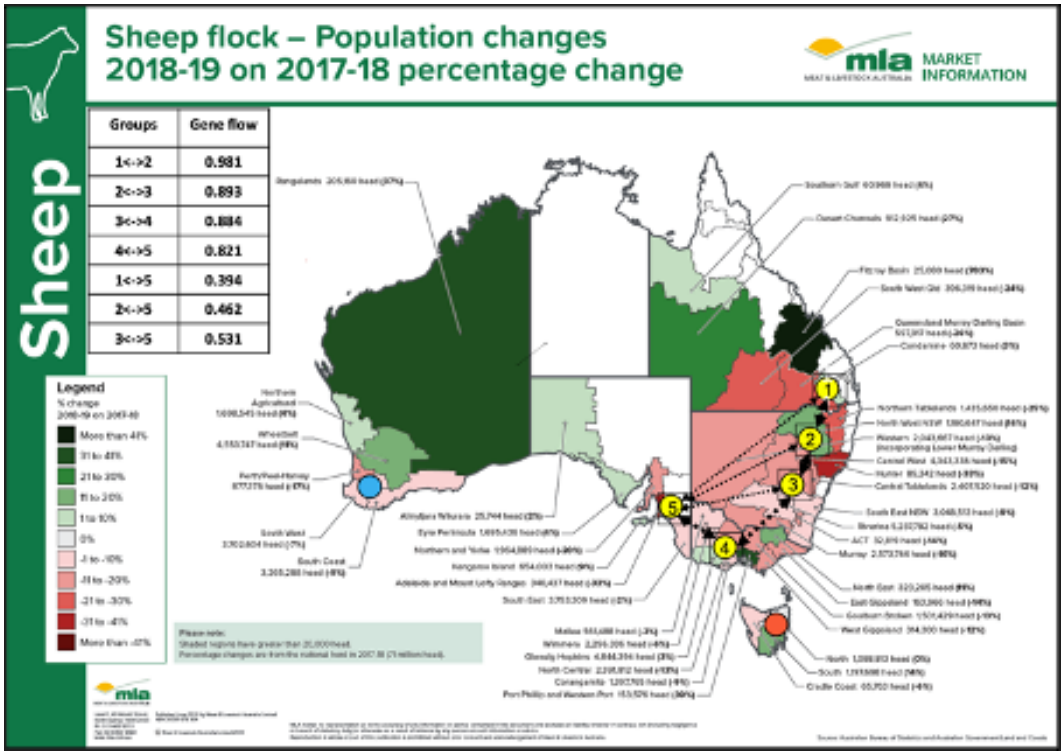


Figure 12. Sheep flock map of Australia showing the sheep population changes (sourced from https://www.mla.com.au/globalassets/mla-corporate/prices--markets/documents/trends--analysis/fast-facts--maps/2020/mla-sheep-flock-population-changes-map-2018-19.pdf) from 2017/18-2018/19 with the migration pattern of *L. cuprina.* The blue, yellow, and red circles represent the clusters of sheep properties from where the samples were collected. The black dotted lines with arrows represent the migration paths/gene flow among the collection sites. The width of the arrows denotes the relative amount of gene flow within the groups (1-5) being explored (that is, the wider the arrow, the more gene flow).

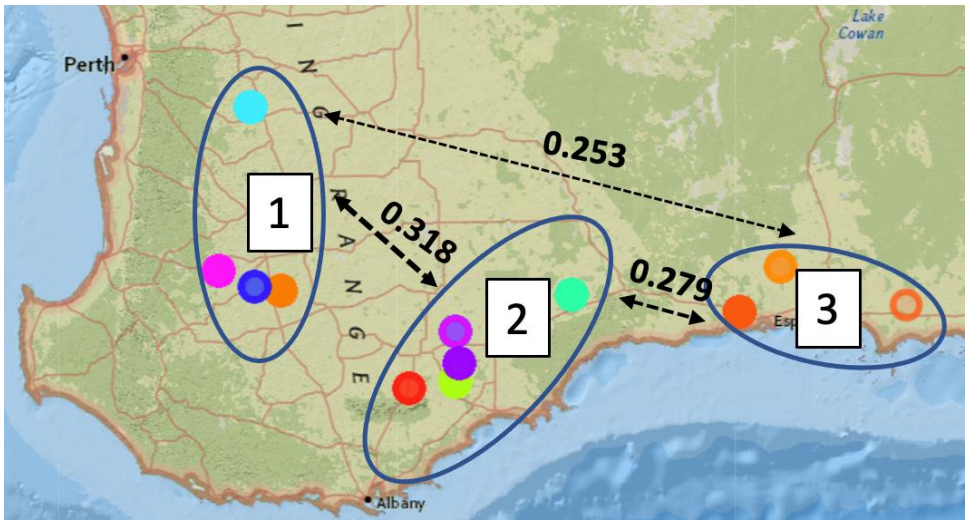


Figure 13. Map of Western Australia showing the migration pattern of *L. cuprina.* The circles 1, 2 and 3 represent the clusters of sheep properties from where the samples were collected. The black dotted lines with arrows represent the migration paths/gene flow among the collection sites. The width of the arrows denotes the relative amount of gene flow within the groups (1-3) being explored (that is, the wider the arrow, the more gene flow). Estimates for the gene

flow rate (Nm) of *L. cuprina* groups in the Western Australian region are shown over the arrows. Bidirectional (mixed) gene flow occurred between the subpopulations in the Western Australian populations.

Though the Tasmanian populations were found to be a genetically distinct cluster from WA and the Eastern Australian populations; based on our data, the subpopulations of *L. cuprina* flies appear to be migrating in both directions and interbreeding between the sheep properties in Tasmania (Figure 14). The gene flow rate (Nm) of *L. cuprina* populations in the Tasmanian region is greater than 1 which means that there are high levels of gene flow between the subpopulations. We found strong gene flow and almost no genetic differentiation between individuals or subpopulations based on 26,277 polymorphic loci. The Tasmanian fly populations are extremely genetically similar to each other and are highly interbreeding which indicates they have likely gone through a population bottleneck. This is something that may be expected if there was a significant drop in the population numbers during a cooler period of the year such as when overwintering. Alternatively, this could be due to a small initial founder event with very few flies entering Tasmania initially prior to their invasion across the state, and then these have differentiated following their arrival and adaptation to local conditions in Tasmania.

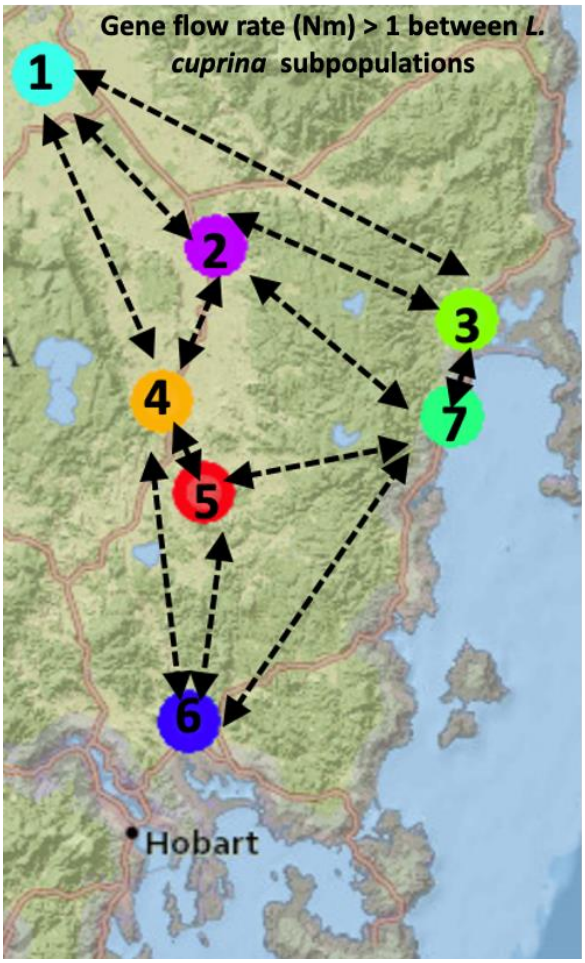


Figure 14. Map of Tasmania showing the migration pattern of *L. cuprina.* The circles 1, 2, 3, 4, 5, 6 and 7 represent the sheep properties from where the samples were collected. The black dotted lines with arrows represent the migration paths/gene flow among the collection sites. Estimates for the gene flow rate (Nm) of *L. cuprina* subpopulations in the Tasmanian region is greater than 1 showing high levels of gene flow. Bidirectional (mixed) gene flow occurred between these subpopulations.

Pool-seq resistance data

Insecticide targets and specific resistance mutations (Table 6) were examined to identify the presence of known resistance alleles. The results from the additional 22 populations showed that no resistance alleles were detected for the populations analysed for major insecticides currently in use. However, we did identify several resistant alleles associated with resistance to insecticides that are no longer in significant use indicating that this approach is valid and is able to detect these changes associated with resistance. Insecticide resistant alleles were detected in the a7-esterase enzyme (*Rop-1* (diazinon) and *Rmal* (malathion) mutations) and the GABA-gated chloride channel (*rdl* - dieldrin). Further details for the resistance mutations analysis are presented below.

Table 6. List of insecticide targets and specific resistance mutations checked/detected in natural population data across all years.

Target	Protein target	Insecticide class	Resistant change detected
GABA-gated chloride channel	Rdl	Organochlorines (Ffrench-Constant, 1993)	Y
a7-esterase	LcαE7	Organophosphates (Campbell et al., 1997)	Y
Voltage-gated sodium channel	Kdr/para	Pyrethroids (Dong et al., 2014)	N
nAChR receptor subunit a6	Lca6	Spinosyns (Perry et al., 2021)	N
nAChR receptor subunit a1	Lca1	Neonicotinoids (Perry et al., 2021)	N
nAChR receptor subunit b2/a8	Lcb2	Neonicotinoids (Perry et al., 2021)	N
nAChR receptor subunit a2	Lca2	Neonicotinoids (Perry et al., 2021)	N
nAChR receptor subunit b1	Lcb1	Neonicotinoids/Sulfoximines (Bass et al., 2011; Perry et al., 2021)	N
Glutamate-gated chloride channel	LcGlucl1a	Avermectins (Wang et al., 2015)	N

***LcαE7* (CVS40_1088)**

We have been able to detect the presence of both the Rmal and the Rop1 resistance allele mutations in the field-collected populations and at different frequencies (Figure 14, Appendix 3).

Diazinon is an organophosphate (OP) that is used to directly control the sheep blowfly. Resistance to this OP is associated with a separate protein substitution to that of *Rmal* in the *LcαE7* gene, a Gly137Asp substitution. In this case, the carboxylesterase activity of the native enzyme is abolished, and a new OP hydrolase activity is conferred on the enzyme, making it more effective against diazinon or OPs including chlorfenvinphos (Figure 15A). In contrast, a different mutation in this same enzyme is associated with resistance to Malathion, a dimethyl OP used to control sheep blowfly. The resistance is due to a point mutation in the *LcαE7* gene which results in a Trp251Leu substitution (Figure 15B). The *LcαE7* gene normally produces a carboxylesterase. The mutation decreases the carboxylesterase activity while improving the enzyme's ability to break down dimethyl OPs, particularly malathion. Protein identity to the lab strain reference sequence is indicated by dots. Those field-collected populations with a higher frequency of susceptible alleles are boxed in green.

The allele frequencies for this site were analysed, and the percentage carrying the *Rop-1* G137A resistant change for each natural population are illustrated in Figure 16. In the 39 genomes scanned, the *Rop-1* G137D resistant change is detected in most of the populations across multiple states (VIC, NSW, QLD, WA and SA) but was not found in the AS collection from Northern Territory, ANE and TUR populations from SA, NWFB, GG and DCKDA populations from NSW, JCO2 from QLD and RH, EL, NPSTBP and CAR populations from WA. 100% of the sequences for the samples from NM, RB, JCHCB and GT populations from NSW had resistance alleles. The high frequency of the resistance allele correlates with the presence of populations resistant to diazinon reported in Sales, Suann, & Koeford, 2020.

In comparison to the variation in frequency of the *Rop-1* allele, the frequency of the *Rmal* allele was 100% in those populations in which it was detected. The resistant allele is present in DCKDA population from NSW, ANENE and TUR populations from SA, AS population from Northern Territory and CAR population from WA (Appendix 3).

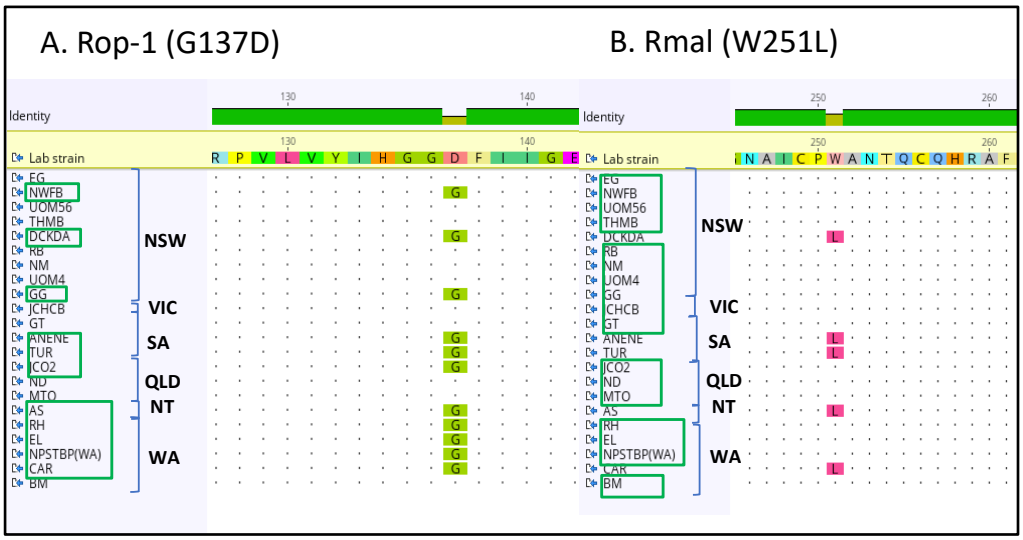


Figure 15. Partial protein alignments of **A.** Rop-1 and **B.** Rmal resistance alleles found in the *LcαE7* (CVS40_1088) enzyme. Panel A shows the G137D substitution that is associated with resistance to diazinon and many OPs (Susceptible#Resistant residue shown). Panel B shows the *Rmal* resistance allele Trp251Leu (CVS40_1088) leading to resistance to malathion. Protein identity to the lab strain reference sequence is indicated by dots. Those field-collected populations with a higher frequency of susceptible alleles are boxed in green.

Figure 16. Map of *LcαE7* allele frequencies for the susceptible allele G^{137} (blue) and *Rop-1* resistant allele D^{137} (orange) that were found for all sequenced field-collected populations (2018/2019-2021/2022).

rdl (CVS40_3522)

Resistant to dieldrin (a historical resistance) encodes a widely expressed ligand-gated chloride channel which mediates the fast inhibitory effects of GABA. It has been shown to have roles in the circuits underlying visual processing, odour coding, learning and memory, sleep and courtship behaviour (Liu, Krause, & Davis, 2007). The critical protein residue is A301 (336 in *LcRdl*) which in resistant strains is substituted to a Serine and confers resistance to dieldrin. Dieldrin resistance became widespread a couple of years after its introduction as a control option in 1955, and its use was discontinued in 1958 due to the development of high frequencies of resistance. The S336 allele was detected in NWFB and THMB blowfly populations of NSW and AS population from Northern Territory (Figure 17). *L. cuprina* populations collected from near, or on, sheep properties from other locations did not show the allele change indicating that the resistance allele frequency has dramatically declined, suggesting that there were significant fitness effects that have led to its decreased frequency in rural populations of *L. cuprina* (Figure 17). There is also a second site in the *Rdl* gene that has been associated with dieldrin resistance. The amino acid M395 is substituted with Isoleucine in the resistant strains. All the *Lucilia* field collected populations were susceptible to dieldrin at this locus. The allele frequencies for the *Rdl* A336S polymorphism identified in natural populations are shown in Appendix 4.

The populations collected from urban areas (2020/2021) of QLD (GB) were resistant). However, this change was not detected in the natural populations). Our analysis of the allele frequencies showed that GB populations were fixed for 100% resistance while the rest of the populations were 100% susceptible.

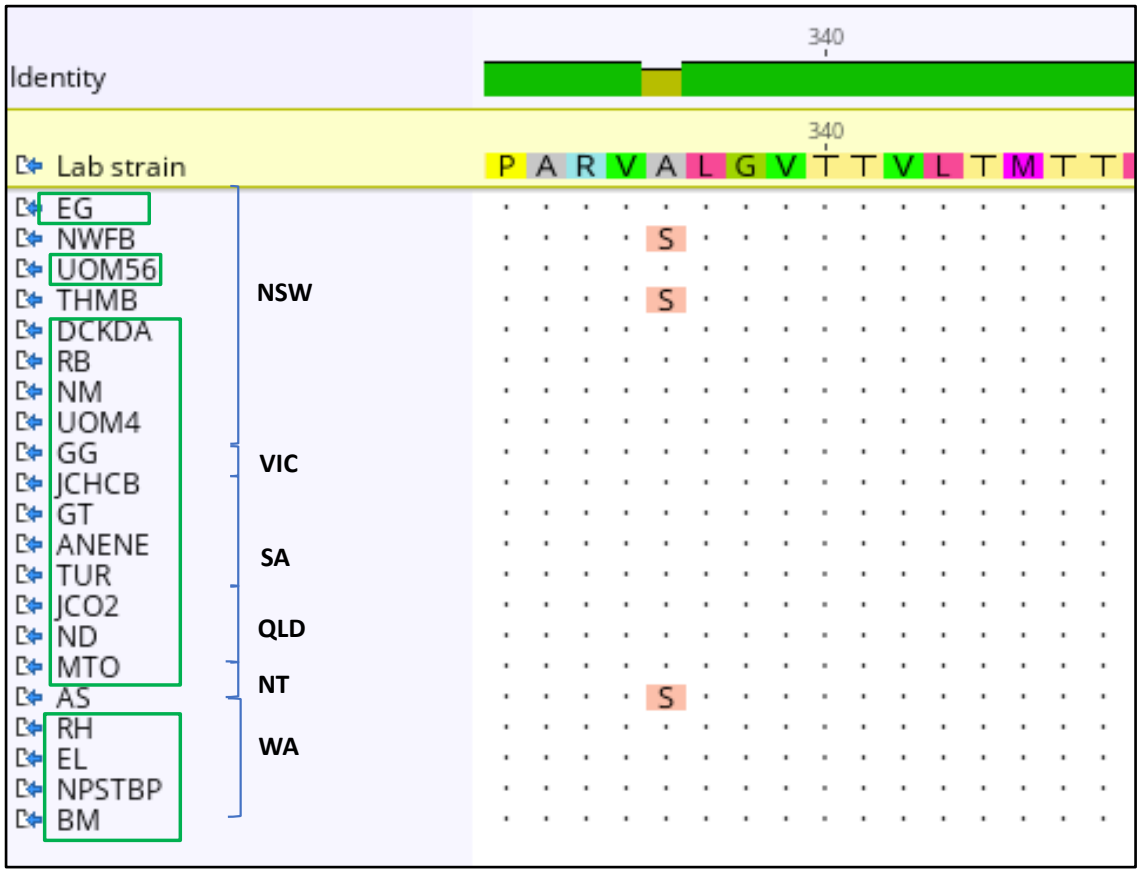


Figure 17. Protein alignment of *Rdl* (CVS40_3522). The Alanine (336) substitution to a Serine residue is associated with resistance to dieldrin. The natural populations detected with the susceptible allele are boxed in green. **Protein Statistics:** Length: 617 aa Identical Sites: 611 (99.0%) Pairwise Identity: 99.9%.

Figure 18. Map of *Rdl* allele frequencies for the susceptible allele A^{336} (blue) and *Rdl* resistant allele S^{336} (orange) that were found for all sequenced field-collected populations (2018/2019-2021/2022).

Summary
The changes that were detected in fly populations which are associated with resistance to compounds no longer in use, including organophosphate (OP) and organochlorine (OC) insecticides, demonstrate that our method can successfully detect the presence of these specific changes and information on their frequency. The frequency of many of the resistance mutations were fixed; however, heterozygosity was detected for the Gly137Asp mutation of the *Rop-1* resistance allele in 50/61 of the natural populations (Figure 15). Resistance to dieldrin, originally detected in the urban Brisbane populations, has now been identified in the field populations of NWFB and THMB from NSW and AS population from Northern Territory (Figure 87).

Proteomics results and analysis.

To achieve the aims of the second objective of this project, a sheep implant trial was conducted to obtain samples of proteins that could be analysed using proteomics. This would allow us to identify proteins that were excreted by blowfly maggots during early stages of flystrike and to detect the response of sheep to the presence of maggots at the skin. Information from this data will provide important insights into what proteins are being used by maggots to initiate wounding and modulate sheep responses, what response is induced by sheep when maggots are present and what proteins are present and hence detectable by the sheep immune system that could support their use as antigens for development of a vaccine to protect sheep against flystrike. Samples were collected from maggots themselves following an initial wash in collection buffer and then after a short incubation in collection buffer for sampling active protein excretion/secretions (from eggs implanted on sheep and on plugs in petri dishes). Samples were also collected from the implant plugs at the sites where no eggs were implanted (mock sites) on the skin of sheep.

We conducted proteomic analysis of 342 samples from the different sample types collected. These samples were processed using single pot proteomics with trypsin digestion. Samples were then purified, and peptide fragments detected using liquid chromatography and mass spectroscopy. The mass spectra underwent peptide identification using MaxQuant comparisons to the sheep and blowfly proteome datasets and label free quantification measuring peptide intensity levels in the samples.

The analysis of different sample types was important given there was a high level of variation in what proteins could be detected from each type of sample. For example, the composition of protein able to be detected in samples from liquid extracted from the implant plugs, those collected from maggots removed from the wound, and samples of excretions/serum collected directly from wound sites varied greatly.

In the excretion/serum samples collected at the skin of wound sites, there was a high proportion of sheep proteins relative to blowfly proteins. This would have potentially masked many of the lowly expressed blowfly proteins present at wounds, however this was overcome through direct sampling of the wash and excretions/secretions of maggots immediately after removal from wound sites. As expected, blowfly proteins were more highly enriched in these samples.

Proteomic analysis of the initial phases of the response of sheep to flystrike.

The majority of the sheep proteins were detected in samples collected from the skin sites where maggots were initiating a strike, although some were identified not only from the wash of maggots collected off sheep, but also in the excretion/secretion samples. Four hundred and ninety-one (491) sheep proteins were present in three or more of the samples collected during the implant study with a core of 85 proteins detected in both skin wounds and from larval wash samples. Through an enrichment analysis of sheep proteins detected in wound samples, we observed several biological pathways that are over-represented which provide some insights into the types of response sheep have during this early stage of flystrike. These included several enriched Gene Ontology (GO) terms as presented in Figure 19 below.

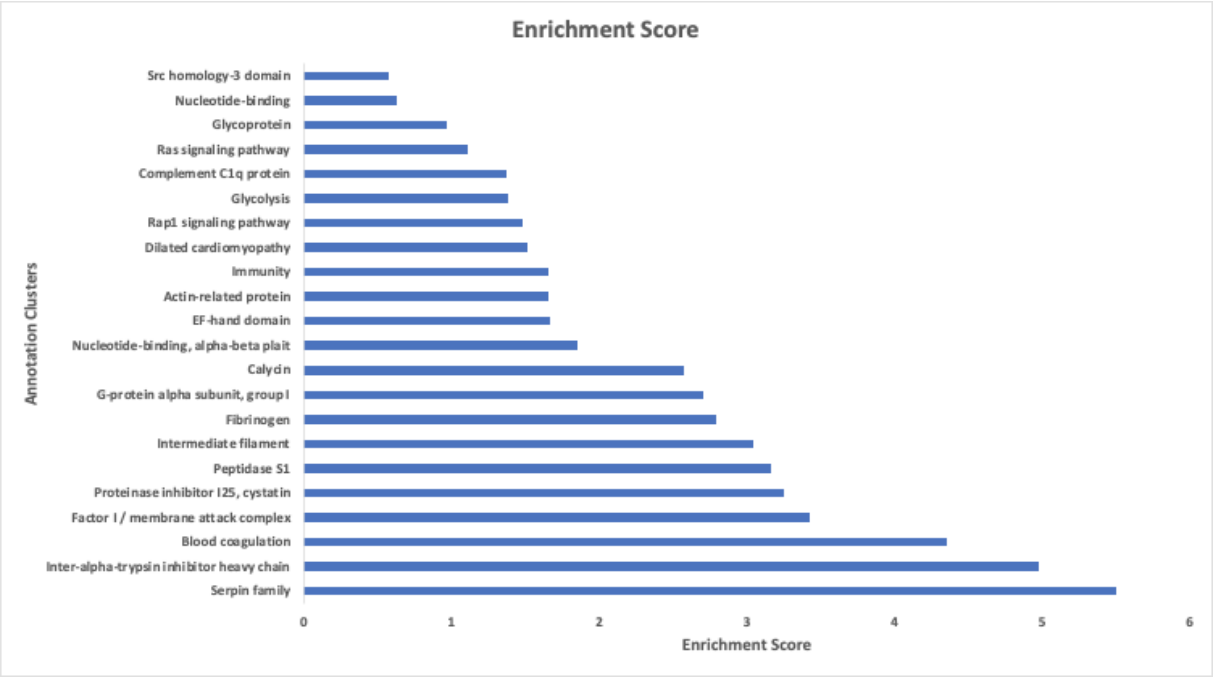


Figure 19. Enrichment scores for the annotation clusters obtained from gene ontology analysis of the sheep proteins identified in samples collected from active early-stage flystrike wound sites during the blowfly implant trials on sheep. We also looked at the proteins which had significantly different representation in samples collected from either the mock skin or the implant skin. Proteins with common biological processes were grouped together providing an insight into the biological functions that are taking place during the wound development (Figure 19). Fewer sheep proteins were found in samples taken from non-wounded skin sites, indicating that these proteins are not present on the skin surface at significant levels.

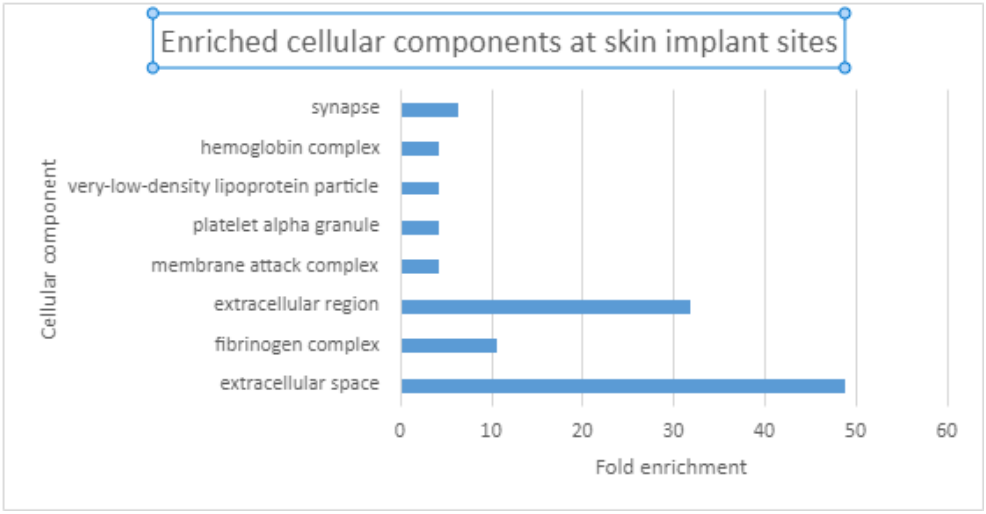


Figure 20. Sheep proteins clustered by biological processes they are associated with, which were enriched in samples from wound sites compared to mock skin implant sites.

There were 47 sheep proteins that had significantly higher levels present in samples from the implant skin (volcano plot figure). Those more highly represented in skin samples are in red, with those with reduced detection levels shown in blue. We detected many proteins that would be expected from a wound site including alpha-2-macroglobulin (can act as a broad spectrum antiprotease), Fibrinogen (can play a role in the early stages of wound repair), Lactotransferrin (can have antimicrobial properties), Plasminogen (precursor of plasmin, a serine protease that can help dissolve blood

clots), Ceruloplasmin (can inhibit the activity of myeloperoxidase during inflammation and infection to protect cells from oxidative stress) and Apolipoprotein A-1 (has a role in anti-inflammatory pathways).

We have also detected proteins from the complement system such as complement C5 and complement C4 precursor that are involved in immune response pathways. Annexin A1, a protein that counteracts the inflammatory pathways induced by innate and adaptive immune responses, and histidine-rich glycoprotein that is involved in the innate immune system of the sheep were also identified.

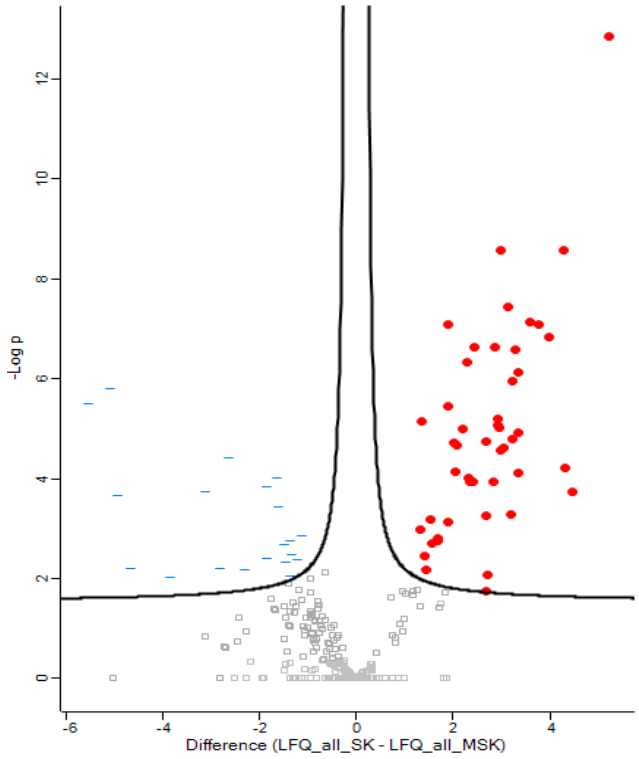


Figure 21. Volcano plot identifying sheep proteins that were significantly different between skin and mock skin implants. Those points above the black line are significant, with those more represented in SK samples in red and less represented in SK samples in blue.

Thirty-eight (38) of these proteins were recognised by the functional annotation as extracellular region proteins which is consistent with the enrichment analysis in Figure 21 above. Further analysis of these proteins suggest that these are part of the response from sheep to the wound formation, with factors associated with both the inflammatory and the immunity pathways present (Figure 22).

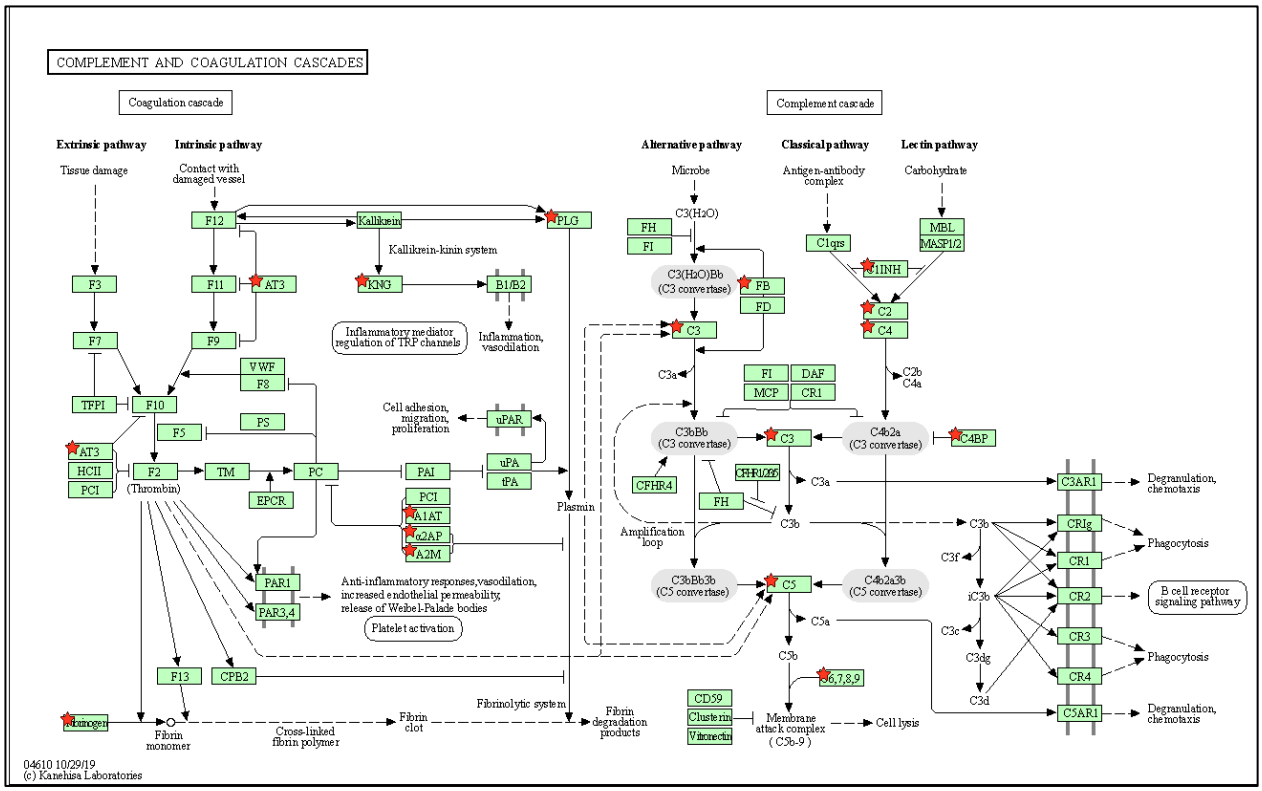


Figure 22. Proteins associated with the complement and coagulation signalling cascades highlighting those proteins identified in this important immune and wound response pathway found in samples at wound sites.

When examining changes in protein levels at different times of the implant study, interestingly we did not detect large changes over time in the level of proteins being excreted by maggots. In particular we did not detect significant differences in these proteins at wounds of different sizes which would suggest that once initiating a wound, there is a consistent protein excretion profile to which the immune system of the sheep would be exposed. This mirrors some of the findings from an analysis of gene expression in a prior project (ON-00397), where it was clear that the location of the maggot is more important than the age of the maggots in terms of gene expression patterns. This makes sense as maggots would hatch depending on these factors and so despite collections at different times, the actual age/lifestage of the maggot is also dictated by various factors that could not be controlled, including temperature at the implant site and the humidity at the site which would vary slightly at each implant point. Developing maggots or those at the early-stage wound have similar gene expression profiles compared to each other, despite different collection times. Only a single blowfly gene was significantly different in samples between the 10am, 2pm and 4pm timepoints, however we observed four sheep proteins with differences which provide some potential insights into the changing processes at the wound over time. The vast majority of sheep proteins identified in these samples are involved in wound response and immunity/inflammation. Below we consider the roles of proteins which were significantly differently represented between the sample collection times, suggesting some changes in the response over time.

The four sheep proteins which had significantly different levels at the 10am and 2pm collection timepoints were ENSOARP00000011651.1, ENSOARP00000008442.1, ENSOARP00000021566.1 and ENSOARP00000007182.1. All of these were more highly represented in the 2pm samples relative to the 10am samples. ENSOARP00000011651.1 is a neutrophil gelatine associated lipocalin, a glycoprotein associated with inflammation. It acts as an acute phase protein and a chemokine involved in innate immunity through limiting bacterial proliferation by sequestration of iron and it can also induce apoptosis through association with a proapoptotic gene, BCL2L11/BIM. ENSOARP00000008442.1 plastin 2 is a protein which acts on inhibition of EIF4EBP1 deactivation, facilitating cell adhesion most likely through cell surface lectins and induction of cadherins. ENSOARP00000021566.1 is a histone H3. Histones are not only associated with bundling DNA in the nucleus. There are also circulating histones that act as damage-associated

proteins providing host defence functions and promoting inflammatory responses. They are a component of neutrophil extracellular traps which help kill bacteria but also trigger inflammatory responses, sometimes through Toll-like receptors or inflammasome pathways. They can also mediate sepsis physiology. ENSOARP00000007182.1 also known as olfactomedin 4, facilitates cell adhesion and can lead to positive regulation of substrate adhesion-dependent cell spreading. Between the 10 and 4pm timepoints, there was a significant change in the representation levels of two sheep proteins and one blowfly protein. This included one blowfly gene, CVS40_8281 (*acp7*, an acid phosphatase with metal binding properties) that was found to be more highly represented in the earlier 10am samples. This might indicate it is present at higher levels early in development. This protein is expressed in cells anterior to the pericardia, a part of the excretory system. It is also possible that at this earlier timepoint there is slightly less sheep protein exudate and so the blowfly protein is more highly represented in the samples.

In addition to the maggot protein, there were increased levels of ENSOARP00000011651.1 and ENSOARP00000008442.1 protein as were observed in the 2pm samples relative to 10am. Other sheep genes were significantly present in this 10am vs 4pm comparison with reduced levels detected in the 4pm samples. One of these genes was ENSOARP00000004002.1 (Haptoglobin) which was higher at 4pm. This protein is involved in the acute phase immune response. The protein ENSOARP00000016806.1 (Beta-2-glycoprotein 1), a negative regulator of blood coagulation, was also significantly reduced. Thus, the reduced relative level of this protein found in the sample at this later time may suggest the wound site is beginning to start the process of coagulation, an expected result. The other protein found at a lower level in the 4pm samples compared to 10am was ENSOARP00000009174.1. This protein is most similar to Factor H from the complement system. This glycoprotein is present in plasma and acts to inhibit the alternative pathway and the amplification loop of the response. Hence the reduction will lead to activation of phagocytosis and potentially lead to further inflammation, so it appears that proteins we observe in the samples are promoting coagulation and inflammation, increasing over time as the wound progresses.

Comparison of sheep selected for breech strike resistance from the New England Merino Lifetime productivity project

One element of the proteomics study was to look at whether there was a difference in the response of two different flocks of sheep to flystrike or in the protein profile of maggots that were establishing strike wounds on these sheep. Of the 14 sheep used in the study, 7 were from the breech strike resistant flock (BSR) and 7 from a non-selected flock sourced from Armidale, New England Merino Lifetime Productivity Project (Dr Jen Smith, CSIRO Chiswick campus). We compared proteins from the sheep wound (which included maggot excretions and sheep proteins) between these different flocks to see if could detect any differences in protein profiles of the wound exudate samples that may be associated with the resistant flock phenotype. We were not able to detect any significant differences between these two flocks. We also examined the protein profiles from eggs implanted on these two flocks of sheep and again saw no significant difference. Our proteomic analysis does not suggest there is any distinction in the response from sheep of either flock, or that there is any alteration in blowfly protein excretions during strike initiation that might be associated with a decreased likelihood of strike. This data matches our findings from histological examination of cellular infiltrate at wound sites, which we outline in a later section of this report.

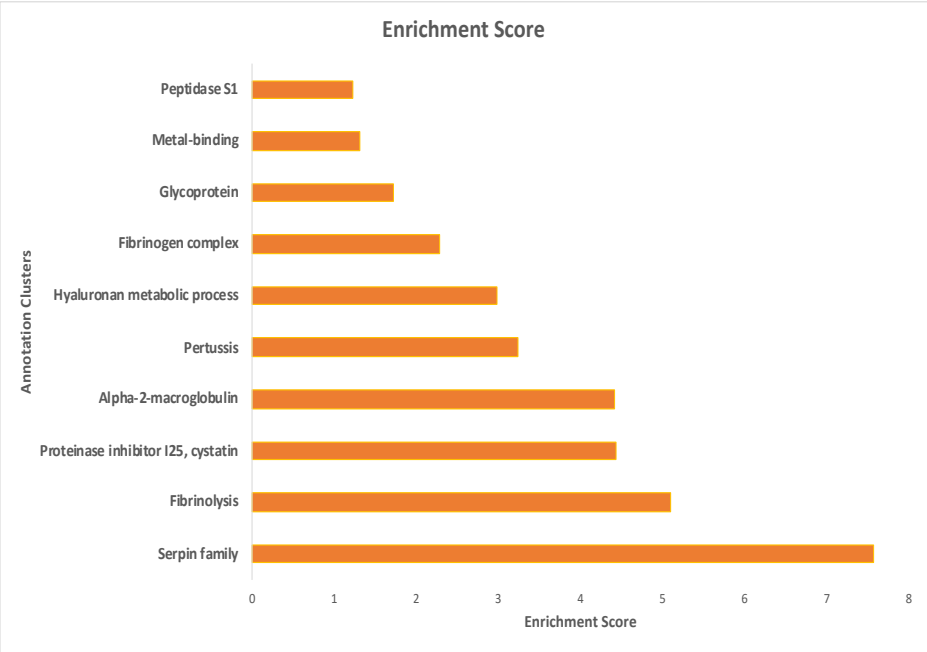


Figure 23. Enrichment analysis of functional groups of proteins that were detected in early maggot wound samples of Breech Strike Resistant and unselected sheep.

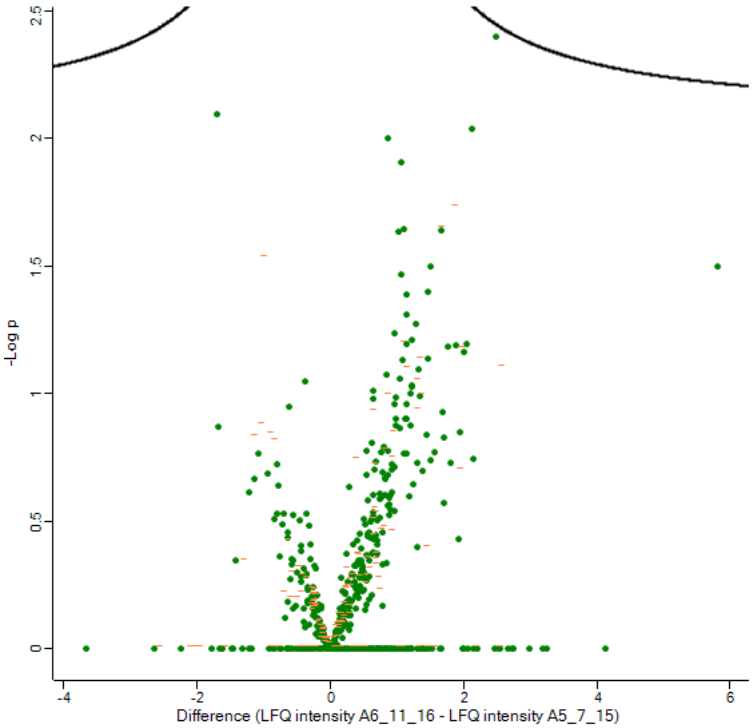


Figure 24. Volcano plot of difference in peptide intensity between the sheep flocks showing that all protein differences were below the significance threshold (black solid line) indicating there were no significant sheep proteins (green) that differ in expression between these two flocks, nor were there maggot proteins (orange) which were significantly different.

Currently, our understanding of what maggot protein will elicit the more effective protective immune responses from sheep is limited. Our study has identified the types of host response from sheep that is occurring during these early stages, however it is not possible to identify what the optimum protective immune response might be. This is because we do not have data on what constitutes a protective strike response. While we attempted to address this through the study by using the two sheep flocks (breech strike resistant v unselected) the results above indicated that it is unlikely that there is a difference in the response of these sheep to flystrike, at least at the protein or cellular levels as far as we can ascertain.

Our findings suggest that other factors are likely responsible for any resistance of sheep from the breech strike resistant flock. For instance, the design of this study is not able to test all phases of flystrike. Prior to the eggs hatching and creating the wound, (the stages examined in this study), adult flies identify a suitable host and select an appropriate site to lay their eggs. These are not factors that were tested in this study due to the fact that blowfly embryos were implanted directly onto the sheep. Hence, factors such as levels of attraction of blowflies to the different sheep flocks that might lead to a reduced propensity of flies to strike particular sheep, could not be ruled out. However, based on our findings, there is no clear biological process that has been selected for in the resistant flock which presents different proteomic profiles during the initial stages of wound formation in comparison to the susceptible flock.

Further work in this area could be performed if strike resistant flocks can be identified whereby which it can be shown that the resistance is associated with an immunological parameter(s) and is not due to other non-immunological factors. Furthermore, these types of studies may prove very useful for studying the immune responses of sheep at wound sites following vaccination. Such studies could be undertaken during current or future vaccine trials to help compare fully or partially protective vaccines to look for correlates with protection through both cellular and/or humoral immune responses.

Proteomic analysis of blowfly proteins produced during early stages of maggot development during flystrike.

In our proteomic study, a maggot protein was recorded as being present in the sample if it was identified by a unique peptide and at least one additional peptide fragment related to that unique peptide sequence. This led to the identification of 764 blowfly proteins detected across all samples. Following this finding we looked at some of the biological pathways these proteins are involved in through an enrichment analysis, which examined how many proteins associated with different biological functions are present by clustering them based on biological functions.

Major biological pathways enriched for the proteins we identified in our samples from maggots at implant sites are presented in Figure 25. There were 13 significantly enriched biological processes highlighted which shows that we were able to detect many proteins being produced by maggots that would be expected to be involved in their growth and development, including an enrichment of stress response proteins, kinases, secreted proteins and energy production related proteins.

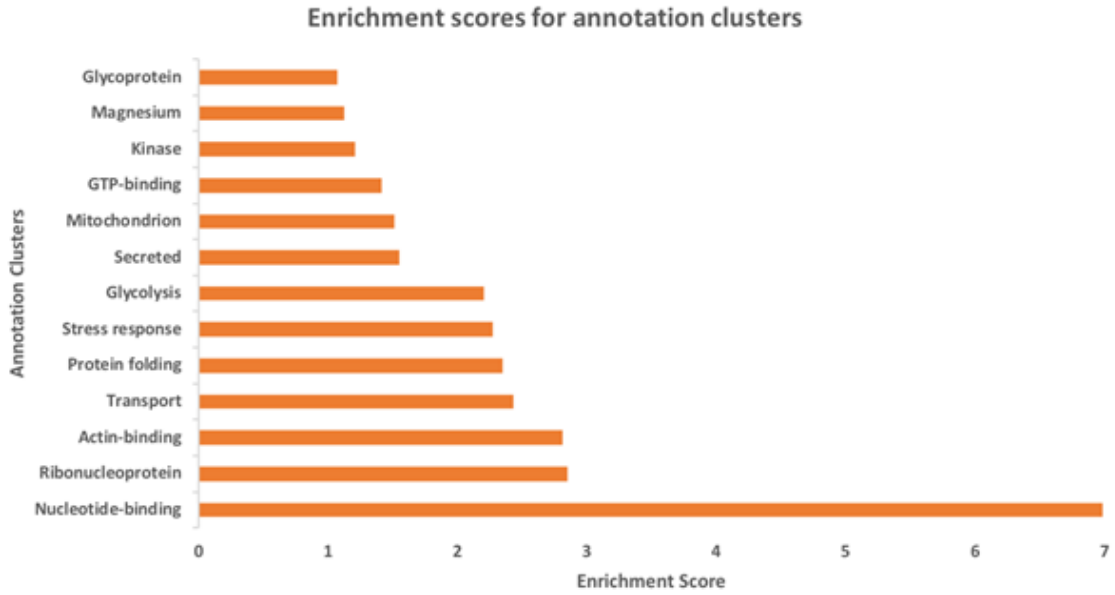


Figure 25. Enrichment scores for the annotation clusters obtained from GO analysis of the proteins detected in larval implant samples.

Of particular interest at the start of this study were proteins that might be excreted as part of the developmental process of maggots in establishing a wound. While there is gene expression data available, this does not confirm whether a protein is secreted or excreted and one of the best ways to conduct this type of analysis is to perform proteomics and identify the proteins that are physically present at the wound site rather than RNA which is being produced in the maggots but may not always reflect protein production or protein levels.

An accurate understanding of what proteins are present is important as these may be the most available for host defence recognition and provide the opportunity for the host to initiate the fastest response to a strike. Bioinformatic analysis of the *L. cuprina* genome predicted 771 secreted proteins and we compared this list to the 764 proteins we have identified in blowfly samples from the study. In total, 98 of the proteins computationally predicted to be secreted across all genes in the blowfly genome were detected as present in the actual protein samples. This might seem a much lower number than expected, however it may be that while some of these are being secreted, it is at a level too low to be detected in our samples. It is also important to consider that these proteins are computationally annotated, and some may not be expressed at this time or in a tissue that will lead to their secretion into the surrounding environment, so would not be detected. Another factor likely to be contributing to the difference between those we detected in our samples were proteins that were not predicted to be secreted. This difference is most likely due to samples accumulating other proteins which would not normally be secreted from dead or injured maggots or from tissue turnover such as proteins lining the midgut forming part of the extracellular matrix but not being actively secreted. This highlights the importance of biologically validating the proteins that are present and reinforces the limitations or caveats which should be placed on relying on the use of bioinformatic approaches alone.

Critically, the results from our sheep implant trial have been highly informative by both revealing the proteins present at wound sites, as well as demonstrating that some proteins which may have been predicted to be present at high levels, are not necessarily detected in the samples. The results demonstrate that regardless of computational predictions of proteins being secreted or not, there is a significant range of proteins outside of secreted ones that are present during early stages of wound formation and potentially available for recognition by host defence responses.

While these maggot proteins could therefore be recognised by the sheep host and potentially trigger an immune response the most important question remains; will use of these proteins in a vaccine lead to a protective host

response? This is something that can only be addressed through further validation work either in the blowfly through manipulation of gene expression, or through trialling them as vaccine candidates in sheep. We present below our assessment of proteins of interest that are based on the proteomics and some computational analysis. At this point it is based around presence and levels of protein present at wound sites. As more data becomes available from other research in this area it may be possible to use an iterative approach to improve the capacity of researchers to predict the most effective protein candidates for eliciting a protective response in sheep against flystrike.

Assessment of proteins and their value for further validation as vaccine candidates.

The research findings from the proteomics research have been integrated with a prior transcriptomics study and an evaluation of protein function to address one component of Objective 2 and utilise data from proteomics to refine the UOM gene lists that were initially based on transcriptomics performed during small scale implant trials in the ON-00373 study. To refine our lists, we used the proteomics results to characterise the proteins present in different sample types and the levels of protein that were found. This provided us with several protein subsets which we used to refine a list of proteins for which further analysis of their role and importance in maggot survival would help validate their potential value for use in vaccine development.

The initial comparison was made between the complete 764 blowfly proteins detected in the study and our prior list of 39 genes identified in the ON-00373 study through transcriptomics. This showed that 15 of the 39 genes of interest from ON-00373 were not detected as proteins, hence these should be considered less attractive as candidates given we did not observe these early in larval development.

We identified 284 blowfly proteins that were observed in three or more of the samples collected indicating they are proteins likely to be regularly observed in these early stages of flystrike. This is something critical for an early response to be initiated by sheep through protein/antigen recognition of the presence of the blowfly maggots on the skin. In addition to these criteria, we also ranked proteins according to their relative amount of protein present in the samples. Proteins were given a ranking based on their peptide intensity with the top 200 proteins in samples allocated a weighting starting at 200 for the highest intensity protein present in a sample down to 1. Using a minimum total cut-off score of 100, this approach resulted in a set of 348 proteins representing the most highly detected proteins in our samples.

These two protein sets of most highly detected and most consistently detected proteins were compared to our earlier gene expression data from early larval development using RNA analysis that included 5016 secreted, transmembrane or orphan RNA transcripts representing 4157 different genes. From this analysis there was an overlap of 102 proteins from the expression data with at least one of these two sets and 56 that were shared between all three. Examining some of the proteins that were not present suggests that many are intracellular proteins that would not be expected to be external to maggots. The presence of these in samples is likely due to some mortality and degradation of maggots during development at the wound that has released these intracellular proteins, or that may have been released during collection and sample processing. Seventeen (17) of the highly expressed potential candidates from the UOM ON-00373 project were also present in all three, with 2 further candidates identified as higher intensity from samples, but these were not consistently detected in three or more sample types (Figure 26).

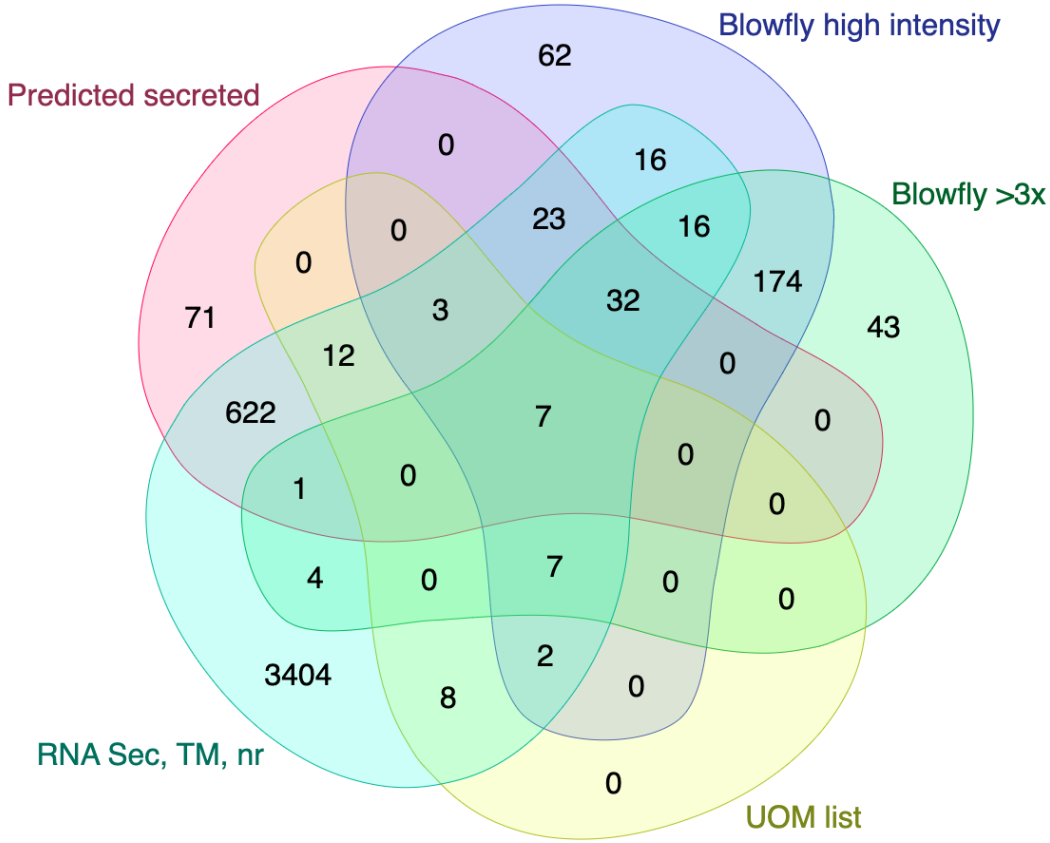


Figure 26. Comparison of the prior UOM proteins identified from the transcriptomics study (ON-00373) with protein sets from this project. The 32 proteins present at the intersection of the three protein and RNA datasets is also being considered of interest given they appear to be regularly present in samples, at a relatively high intensity and that they are predicted to be secreted.

Whether a higher protein level would be the optimal characteristic for a vaccine candidate remains debatable. The higher level of protein expression might suggest these proteins are important for larval development or establishing the wound and that there will be significant amounts of protein available for host defence to detect and respond to. The possibility of it being a less favourable characteristic should also be considered as a large amount of protein may also indicate that even if there was a strong immune response, this may not be of a high enough magnitude to negate the effect of the antigen and impact the function of the protein at a level sufficient to kill maggots. There may be some value towards protection if it can disrupt some function through impairing maggot development, however in these cases the candidates would likely be more suited for use in combination with other antigens (multivalent vaccines) where the components may synergise the effects of each other on maggot development and survival sufficiently to prevent the maggots from maintaining a wound at the strike site.

Histology results and analysis

Results from the histological analysis noted a significant influx of cells into the skin at the site of blowfly infections compared to the uninfected control sites. This influx was in the form of CD4+, CD8+, gamma-delta+, T19+ T cells as well as B cells and CD1+ Langerhans cells. There was, however, no significant difference in the cellular infiltrate that was recorded between the sheep that had been selected for breech-strike resistance compared to those animals from the non-selected flock. These results might indicate that the expression of resistance in the breech-strike resistant flock is not specifically associated with a particular influx of any one or other cell types. However, what is not clear from this analysis is whether specific cell types are contributing to the resistance based on what mediators they could be producing (for example specific cytokines). Subsequent analysis of these factors may correlate this resistance to a mediator produced by a specific cell type; however, at this stage this hypothesis has not been investigated.

7. Discussion

Our study has been able to provide evidence that would support a moderate gene flow within the three population clusters defined in this survey. However, it will be important to examine the distances individuals are able to travel, particularly at borders between these clusters (eg. WA/Eastern Australia) to obtain a greater understanding of the ability of flies to migrate and mate successfully across to other populations. Identifying how prevalent migration is will be critical for assisting the design of resistance management plans which aim to prevent the spread of insecticide resistance. At a local level, the movement of flies between areas based on release and recapture has been previously reported as quite limited. Those migrating individuals recaptured travelled distances ranging up to 4.7 miles (~7.5km) over <30 hours (Gilmour, Waterhouse et al. 1946). Our analysis shows that despite the expanse of the east coast population cluster, moderate levels of gene flow are occurring, and this could lead to a fast spread of resistance. The capacity for resistance to spread in Australian sheep blowfly populations is supported by the historical spread of dieldrin resistance. There is also the possibility resistance is currently spreading through gene flow across different blowfly populations in the case of cyromazine and dicyclanil resistance. This is a significant issue for the sheep industry, with the spread of resistance to these two major actives concerning given products containing them have the ability to provide longer-term fly control than many alternatives. Resistance was reported on sheep properties in NSW around the turn of the century (Levot 2001). The presence of cyromazine resistance was confirmed in the lab and a 2014 survey found cyromazine resistant populations were present at low levels (62% of total samples cyromazine resistant, 14% of samples cross resistant to cyromazine (greater than a discriminating dose but less than 8-fold) and dicyclanil) (Levot 2014, Levot, Langfield et al. 2014). More recently resistance has been observed at much higher rates, 10/10 samples collected in 2017/18 had high levels of dicyclanil resistance (LC50 from 13.4 to 46.5 fold) with these strains also cyromazine and ivermectin resistant (Sales, Suann . 2020). This is potentially a much wider issue given 100% of the 55 samples from NSW were resistant to both dicyclanil and cyromazine while of the 100 samples tested nationwide, 73 were cross resistant and only 12 susceptible to both compounds (Sales 2020). Results from this study provides significant advances in our understanding of blowfly population structure which should aid further studies into the potential for resistance to spread within and between populations and in assessment of the feasibility of introducing area-wide control strategies and predicting their likely effectiveness on different population clusters. Given there is significant population structure, this would suggest there is merit in a closer examination of the potential for area-wide strategies to tackle certain populations, particularly those in WA and Tasmania. The results from this study can provide valuable data that can be used to model some of these strategies and inform aspects of their design. However, further work to better understand the conditions that favour or anthropogenic reasons that underlie possible migration paths/events between the southeast and WA populations in particular is required to ensure that reinvasion would not readily occur. Some other barriers to gene flow and admixture between these populations also warrant investigation to look for differences in these fly populations that may be inhibiting effective gene flow, such as endosymbionts like *Wolbachia*, chromosome inversions or other biological factors in addition to geographic distance and climate.

Utilising the proteomic data generated in this study, we have been able to identify a subset of proteins which are consistently expressed by maggots during their early stages of development and are relatively high in their expression levels during flystrike compared to other blowfly proteins. To prioritise those for further investigation we have also considered whether the proteins are likely to be secreted and if the gene encoding the protein is highly expressed in the earliest larval stage. This has resulted in an additional set of 32 proteins being added to our potential candidates of interest. Our research has also helped identify 20 proteins which we would no longer consider of high priority given they were not detected in the proteomic analysis. We have however not completely ruled these maggot proteins out, as it is still possible that they are present but at a level below our limit of detection.

8. Impact on Wool Industry – Now & in 5 years' time

We believe that our commitment to CSIRO to share our data that they have used to evaluate biological aspects of their vaccine candidates is of immediate benefit to their blowfly vaccine program. We have provided CSIRO with detailed protein sequence data relating to the sequence variation of their candidates from samples collected from different regions of Australia. This information was provided to help them evaluate whether their potential vaccine candidates will have broad efficacy across Australian sheep regions to offer protection to sheep from flystrike. Furthermore, our data from the proteomics study has also confirmed the excretion/secretion by blowfly maggots of some of CSIRO's main vaccine candidates.

The need to control flystrike, and the root cause of this problem, blowfly populations, has both immediate and long-term aspects which will be improved through the results of this research. Our research has enabled us to define three distinct blowfly population areas that provide the most comprehensive data thus far on how blowflies move between areas. With recent concerns about insecticide resistance, particularly the threat to long-term efficacy of dicyclanil and cyromazine-based fly protection products, our population findings can be integrated into resistance modelling, providing empirical data that should help improve the accuracy of these models which are being used to advise growers on how to best manage their use of insecticides to control blowflies. Given we identified gene flow within these populations, the implications for current insecticide resistance management programs is that broader scale coordination could be beneficial. This is because the spread of resistance alleles through blowfly populations may be able to occur at a higher frequency than expected. Alterations to usage recommendations for any particular chemical for which control failures or reductions in efficacy are being reported should be considered in this broader context. Our results will contribute to developing more refined blowfly resistance models and the improved recommendations from the models will assist growers to better manage their chemical usage and help sustain the efficacy of the limited chemicals available for blowfly control. The data from our study can also assist efforts towards evaluating and designing area-wide insect control programs such as Sterile Insect Technique (SIT) to control *L. cuprina.* The eradication of blowfly from isolated regions, and potentially larger areas could be investigated based on this new knowledge of population structure.

Future impact

There is a strong desire of the sheep meat and wool industries to reduce growers' reliance on mulesing as part of their blowfly control practices. This change obviously needs to be managed in a way that avoids a decrease in animal welfare through increased flystrike prevalence/reduced effectiveness of control measures will be key to industry uptake of any new practices. Constant refinement and improvements of blowfly management practices are required to ensure that control options such as insecticides, remain effective and that new information on best practices are integrated into flystrike management plans to achieve this. The outcomes from this research will provide valuable insights to help maintain and revise existing control options for not only current, but also any newly released insecticides to better protect and prolong their effectiveness. The fundamental population genetic data derived from this project will provide a platform for future research in this field. The data can be mined using new knowledge of resistance alleles and can be used to compare changes in allele frequencies between the current dataset and future sample collections which has important implications for managing insecticide resistance. For example, this data can be used for tracking the movement of blowflies and determining their origins which can be used to identify possible routes of migration that may allow the spread of resistance to be prevented or slowed down through the use of appropriate biosecurity measures. As stated previously, results from the proteomic study are assisting current efforts towards a vaccine against flystrike and will provide an ongoing resource for studying this parasite and developing novel blowfly control solutions.

9. Conclusions and Recommendations

Conclusions

The genomics approach applied in this study has been able to demonstrate that there are three distinct populations of *L. cuprina* in Australia. These populations consist of flies located in the eastern states and South Australia, Western Australia and also a population in Tasmania. These are likely to be separated through the significant geographic barriers between these regions. Our study has provided significant insights into likely movement of blowflies within these population regions and offers the potential to improve modelling of fly movement to better predict the likely spread of resistance if it arises. By taking into account this movement, more effective insecticide resistance management plans can be developed and adopted. The application of our research findings is already underway to help improve the current flystrike decision tool available at the ParaBoss website that is widely used by sheep growers. Further outcomes from genomic analyses addressed the genetic differences in the NB flies compared to field populations that we identified (Results section and Appendix 2). Based on these results we have discussed with CSIRO the need to ensure their test strain remains field relevant for their trials. To reduce the impacts of continual lab rearing and associated lab adaptation that may be occurring, ideally freshly collected *L. cuprina* field samples will be regularly introduced to the NB population.

The proteomics approach has been able to identify many proteins that blowfly maggots excrete during flystrike wound initiation. This has resulted in the creation of a list of candidate proteins that may assist in the development of a vaccine to protect sheep against flystrike. While further validation is required, there are many proteins we identified in our study which are not within the scope of the current CSIRO vaccine program. These novel candidates may provide avenues to enhance the efficacy of current prototype vaccines or form the basis of more efficacious vaccines in their own right.

In agreement with our analysis of proteomes from the susceptible and breech strike resistant sheep flocks, we were not able to identify clear differences in different cell populations present at the site of wound initiation. Hence, the mechanisms by which these sheep may be more resistant to flystrike remains unclear, but we would hypothesize that it is probably not due to innate responses by the sheep host. There are several stages during the initiation of flystrike which could be contributing to the resistance, however the implantation of blowfly eggs directly on the sheep meant that factors such as host/odour detection or egg-laying site preference of blowflies were not examined.

Recommendations

1. Develop a strategy to preserve the data and resources from this project as a resource for future studies.

This study has resulted in significant insights being gained into blowfly biology that will provide ongoing value to research into control of *L. cuprina*. Through the detailed analysis of blowflies from different regions, it has demonstrated the population structure that exists which can be exploited to develop area-wide management programs and improve insecticide resistance modelling. To do this it is important that the data is available for any future work and one major recommendation is that the genomic sequencing data be stored, or permission be granted for UOM to submit the data to online databases. We would also recommend links to details of the data availability be added to the ParaBoss website and links to the data itself be created so access is readily available to any researchers that are working or considering research in this space. For example, this type of database could be updated with any population surveys that may take place in the future. However, a broader goal of maintaining and curating AWI's datasets from all funded projects would also be valuable to consider.

In addition to the genomic data, there are many biological samples that could be of use for future projects. The blowflies collected could provide a snapshot of the allele frequencies in field populations that can be examined in future if an insecticide resistance gene is identified. These samples need to be maintained in freezers which would

require a small funding commitment from AWI to enable their ongoing maintenance. In addition, it is recommended that AWI consider the value of any future field surveys of blowfly submissions that it commissions to retain some samples to establish a repository of blowfly samples which are available for further study as and when needed. Potential uses of these samples would be to aid research into new insecticide resistance development and spread in the field, or for example, if there are major changes in how blowflies impact sheep, such as an increased severity of flystrike wounding.

2. Integrate the genomics and population data with future insecticide resistance studies and conduct further analyses to extract further value from these large multi-season datasets.

Given the increasing threat of insecticide resistance to effective control and prevention of flystrike the data should be used to help inform resistance modelling. This is a recommendation that will be pursued through the recently commenced AWI managed Informed Modelling of Sheep Blowfly Chemical Resistance project through collaboration with the University of Tasmania (Brian Horton) and NSW Department of Primary Industries (Narelle Sales). This project is expected to improve predictions of the potential for resistance to spread in the field. The genomic data can also be used to examine other insecticide targets if new insecticides were to be registered or developed for control of flystrike. Enhancing the availability of the datasets from this and other projects will also assist in encouraging important research into blowfly biology and stimulate projects for PhD students which may lead to new insights into novel pathways to improve blowfly control practices.

Along with the current short and long read data, a further upgrade can be made to the Australian blowfly reference genome by sequencing the genome to a chromosomal level assembly. For any future genomics/population genetics work, the chromosomal level assembly of Australian sheep blowfly will provide researchers with a high quality and robust reference genome. A chromosomal reference genome of American strain of blowfly (GCA_022045245.1) is already available online but this genome only aligns to 96% of the Australian sheep blowfly genome. The chromosomal level assembly will show a marked improvement in localization and visualization of genetic diversity that will be beneficial for any future population genetics work such as Genome Wide Association Studies, linkage disequilibrium and fine mapping studies. Long read sequencing (PacBio HiFi reads) data for different field populations of *L. c. dorsalis*, *L. c. cuprina* and *L. sericata* can also help in finding gene families involved in parasitism, adaptation and invasion mechanisms of blowflies. With the help of the datasets already generated and the prospect of new datasets, a bioinformatic analyses can be done to study genome inversions which may have created a series of physical differences between the species. Further bioinformatic analyses of the genomic data can shed light on the hybridization between *L. cuprina* and *L. sericata*. Frequent and recurring hybridization raises the possibility of horizontal transfer of resistance genes in/between sheep growing properties and can be monitored in the field The current/ newly sequenced genomic data can be analysed to expand on the GWAS to find alleles associated with new/ existing insecticide resistance in the field.

3. That the list of potential vaccine candidates identified in this project be further assessed to determine their role in maggot survival through validation in the fly.

This study has identified many proteins present during initial stages of flystrike, many of which are not well characterised, and we would also recommend considering further investigation the role that these proteins might play in maggot survival and development. This could be assessed through the use of RNAi to selectively knockdown the activity of these proteins. The identification of maggot proteins that when inhibited lead to larval mortality could then be subsequently assessed as potential vaccine candidates using the sheep vaccine and challenge system.

4. That AWI consider additional strategies for example *Wolbachia* to help control the sheep blowfly

Despite significant efforts to control the sheep blowfly major challenges remain. In order to provide improved control, it is clear that a multipronged approach will continue to be needed and therefore it is recommended that AWI continue

to invest in new potential control strategies. One area that we consider is worth investigating is in the area of *Wolbachia,* its ability to infect blowflies and potential for its use to control blowfly populations. *Wolbachia* has already been used as part of area-wide insect control strategies to control mosquito populations on multiple continents and the application of this approach to other insect pests is being pursued. We know that blowflies can harbour *Wolbachia* infections as they have been previously identified from the field collected blowfly samples from across Australia. Because of the various effects of *Wolbachia* on host reproduction, sex ratio, feminism and cytoplasmic incompatibility the research on *Wolbachia* could lead us to new biological control strategies for flystrike.

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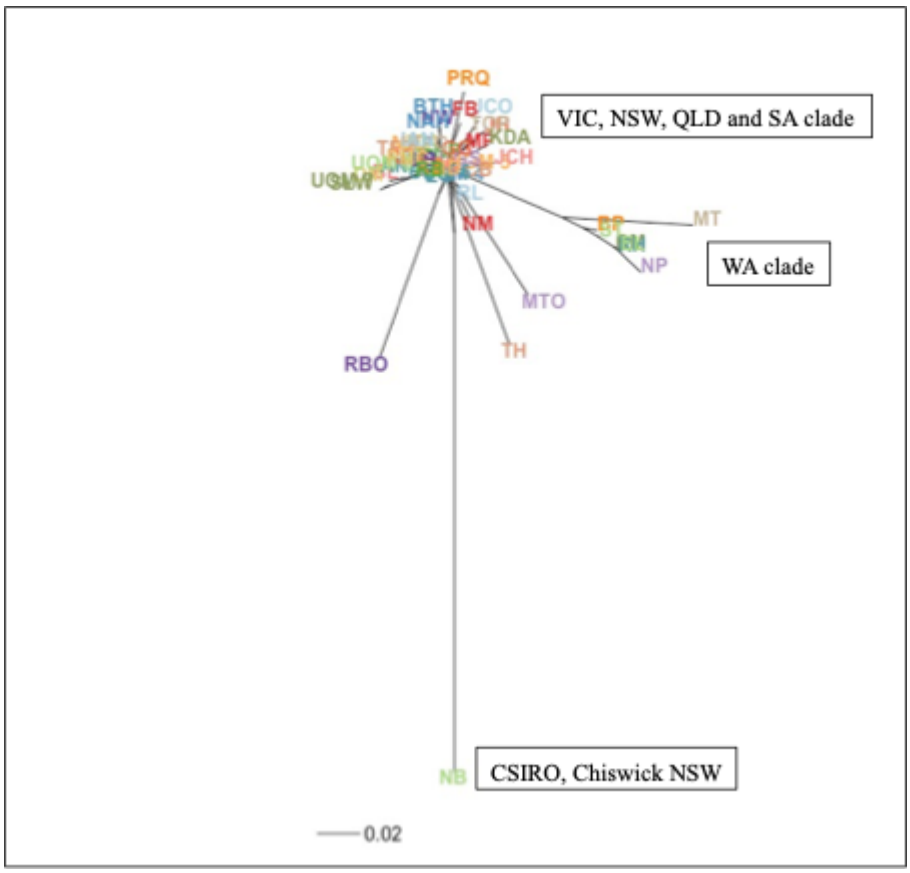
11. List of Abbreviations and/or Glossary

NGS – Next generation Sequencing.
DArTSeq – Diversity Arrays Technology Sequencing. A genotyping by sequencing system that uses NGS to identify informative representations of variation in DNA samples.
PoolSeq – Pool Sequencing.
GO – Gene Ontology.

Appendix 1. Statistics of sequencing data for *L. cuprina* and *L. sericata*

Sample name	Total reads	Mapped reads	Mapping rate (%)	Average depth(X)	Coverage (%)	Total SNPs
Lucilia cuprina						
CAR	173,996,216	168,541,377	30.69	30.69	83.34	2,964,086
NWFB	181,209,618	169,310,761	93.43	41.95	84.35	3,880,675
NPSTBP	159,140,034	139,019,732	87.36	35.01	83.78	3,429,208
JCHCB	166,315,904	147,031,497	88.40	35.78	84.18	3,497,796
NM	169,363,488	129,003,757	76.17	31.69	83.78	3,394,984
UOM4	153,602,692	150,041,856	97.68	30.17	83.84	2,618,043
ND	164,574,098	152,594,016	92.72	31.94	84.05	2,863,844
RB	172,272,160	132,000,545	76.62	39.11	83.21	3,612,042
BM	160,362,610	157,168,320	98.01	30.12	83.37	2,386,164
RH	154,339,126	151,261,431	98.01	29.93	83.02	2,039,632
JCO2	172,400,262	164,769,832	95.57	43.41	83.95	4,013,823
EL	154,729,750	148,260,210	95.82	35.16	83.65	3,591,341
GT	173,548,688	165,939,692	95.62	45.53	83.97	4,021,860
EG	162,980,460	159,777,529	98.03	31.20	83.77	2,338,904
GG	178,012,490	170,112,035	95.56	46.02	84.06	4,021,217
AS	158,621,264	153,989,539	97.08	33.48	83.61	3,300,431
MTO	166,010,616	133,419,131	80.37	35.37	83.60	3,681,493
DCKDA	163,539,038	134,626,890	82.32	33.75	84.10	3,511,442
TUR	157,068,540	152,592,174	97.15	34.47	84.02	3,227,940
THMB	180,965,750	160,923,080	88.92	39.79	84.20	3,539,705
ANENE	155,263,310	124,816,340	80.39	31.63	83.95	3,367,640
UOM56	156,245,864	152,912,014	97.87	29.93	83.58	1,854,100

Appendix 2. Analysis of NB flies (CSIRO trial blowfly strain) for genetic similarity.



Neighbour joining tree (unrooted) visualizing genetic similarity among the different populations of *L. cuprina* which highlights the difference in the genetic similarity of the NB flies compared to other blowfly populations.

Appendix 3. Allele frequencies for the Rop-1 G137D polymorphism identified in natural populations.

Population	% Susceptible allele	% Resistant allele
UOM56	7.14	92.86
EG	6.24	93.76
THMB	10.36	89.64
RH	79.12	20.88
NPSTBP	82.09	17.91
JCHCB	0	100
GT	0	100
DCKDA	72.66	27.34
UOM4	8.80	91.20
TUR	100	0
MTO	6.47	93.53
RB	0	100
NWFB	75.98	24.02
NM	0	100
ND	7.51	92.49
JCO2	55.38	44.62
EL	66.12	33.88
GG	76.87	23.13
BM	19.81	80.19
AS	100	0
CAR	100	0
ANENE	78.61	21.39

Appendix 4. Allele frequencies for the Rdl A336S polymorphism identified in natural populations

Population	% Susceptible allele	% Resistant allele
UOM56	100	0
EG	85.71	14.28
THMB	20	80
RH	100	0
NPSTBP	100	0
JCHCB	100	0
GT	100	0
DCKDA	100	0
UOM4	100	0
TUR	93.75	6.25
MTO	100	0
RB	100	0
NWFB	20	80
NM	100	0
ND	91.42	8.57
JCO2	100	0
EL	100	0
GG	100	0
BM	100	0
AS	0	100
CAR	100	0
ANENE	100	0