Final Report to the Foundation for Rabbit Free Australia

January 31 2016

"Investigating rabbit (*Oryctolagus cuniculus*) resistance to rabbit haemorrhagic disease virus (RHDV) variants in Australia"

Amy Iannella, Phillip Cassey, Nina Schwensow and David Peacock – University of Adelaide

Executive Summary

We have examined the factors contributing to survival and reproductive success of rabbits, with a view to identifying targets for new control activities. DNA was successfully sequenced from 401 rabbits from a South Australian population, and used to determine familial relationships between the rabbits. Breeding success appears to be concentrated in older (and likely more dominant) rabbits, with greater chance of survival for rabbits born in July-October, while natal warren had little effect. Further analysis of this dataset is being undertaken to tease apart the relative contributions of parentage and birth-date on rabbit survival, and the relative contributions of social dominance and genetics to the parentage effect.

Although this is nominally the 'final report' from this project, the rich genetic dataset created here is suitable for analysis in further depth, as well as with a greater breadth of focus. Through my PhD I will to continue reporting findings generated from this data (in published international scientific journal articles and popular media) as they occur.

Project Rationale

Recent increases in rabbit numbers around Australia (for example see the paper by Mutze et al. in *Wildlife Research* 2015) have prompted concern about the rising rabbit genetic resistance to RHDV, which was identified by Peter Elsworth and colleagues in 2012. Subsequent research has focussed heavily on methods for circumventing this resistance, however the actual degree to which genetic resistance to RHDV is impacting on rabbit populations is still unknown.

The aim of my project is to determine how much impact genes are having on the rates of rabbit survival and reproduction, as compared with other contributing factors like climate or the abundance of insects, which spread disease. I am doing this by using DNA to create a family tree for all rabbits in a large South Australian colony and then comparing the rates of reproduction and survival between related and unrelated individuals. If new genes are having a large impact on rabbit populations then we expect to see a big difference in survival between different rabbit families, if the impact of genetics is small compared to environmental factors then we expect to find that survival rates are very similar across all rabbit families.

One hypothesis of particular interest, which I am testing, is the effect of RHDV timing on the survival of rabbit kittens. Because very young rabbits have an innate resistance to RHDV, those who are

exposed to the virus at less than 6 or so weeks of age will usually survive (irrespective of whether or not they have resistance genes) and go on to become immune for the rest of their lives. This study will show whether most rabbits that survive to become breeding adults are more likely to do so because of resistance genes or because of early exposure to RHDV. If the timing of RHDV exposure is having the largest impact then further emphasis should be placed on the appropriate timing of baiting to seed RHDV outbreaks. On the other hand, if genetics is playing the largest role in helping rabbits survive to reproductive age, then the development of new RHDV strains to counter genetic resistance will be vital.

Findings

Reproductive success was distributed unequally among mature rabbits, which is consistent with literature recording a significant dominance hierarchy among rabbits. Of 90 mature males and 62 mature females, only 43 and 42 respectively were found to have offspring in this study. Furthermore, only half of these parents (25 sires and 24 dams) had any offspring that survived to themselves reach reproductive age. Although the extremely high mortality of kittens is well recorded, the parentage data generated by this sequencing project suggests that surviving offspring can predominantly be attributed to a select few adults, illustrated in Figure 1. These parents tend to be the older rabbits present on site, and as such are likely to be socially dominant individuals.

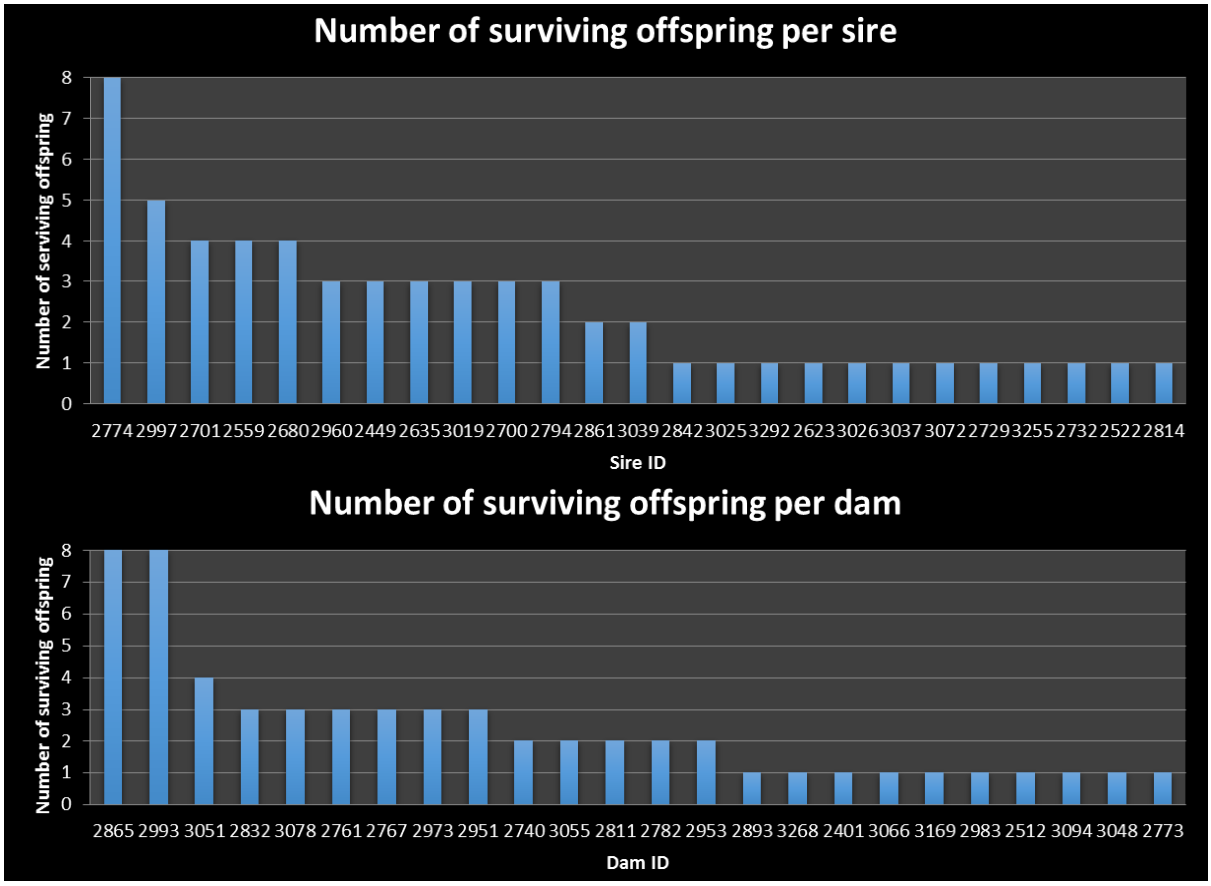

Figure 1: Distribution of surviving rabbit offspring among sires and dams from Turretfield Research Station in 2013 and 2014. Only parents with at least one offspring surviving to maturity are shown.

No strong trend was observed in the proportion of surviving offspring based on the natal warren. Smaller warrens such as bt and 6A (See Figure 2) do appear to have slightly lower rates of kitten survival, however the statistical significance of this is yet to be tested, the weak trend could be an artifact of lower sample sizes in most of these warrens.

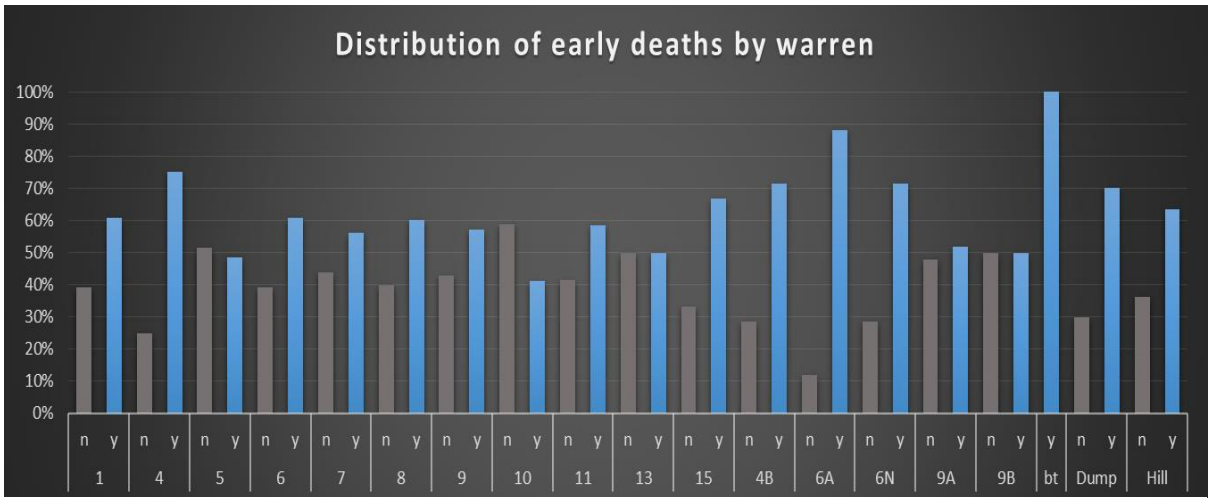

Figure 2: Distribution of rabbit deaths at Turretfield Research station in 2013 and 2014 by warren. Blue bars indicate proportion of rabbits dying before sexual maturity, grey bars indicate proportion of surviving offspring for each warren.

Survival rates of kittens do appear to fluctuate significantly according to month of birth, as illustrated in Figure 3, with almost all kittens born between November and May perishing before they reached reproductive age. This observation is consistent with impacts of both seasonal climate and resource availability and seasonal disease outbreaks. To tease apart these effects a more detailed analysis will be performed, analysing each year individually based on exact RHDV outbreak timing and climatic conditions. This will enable testing of the hypothesis that rabbit age of first exposure to RHDV is a significant factor contributing to survival.

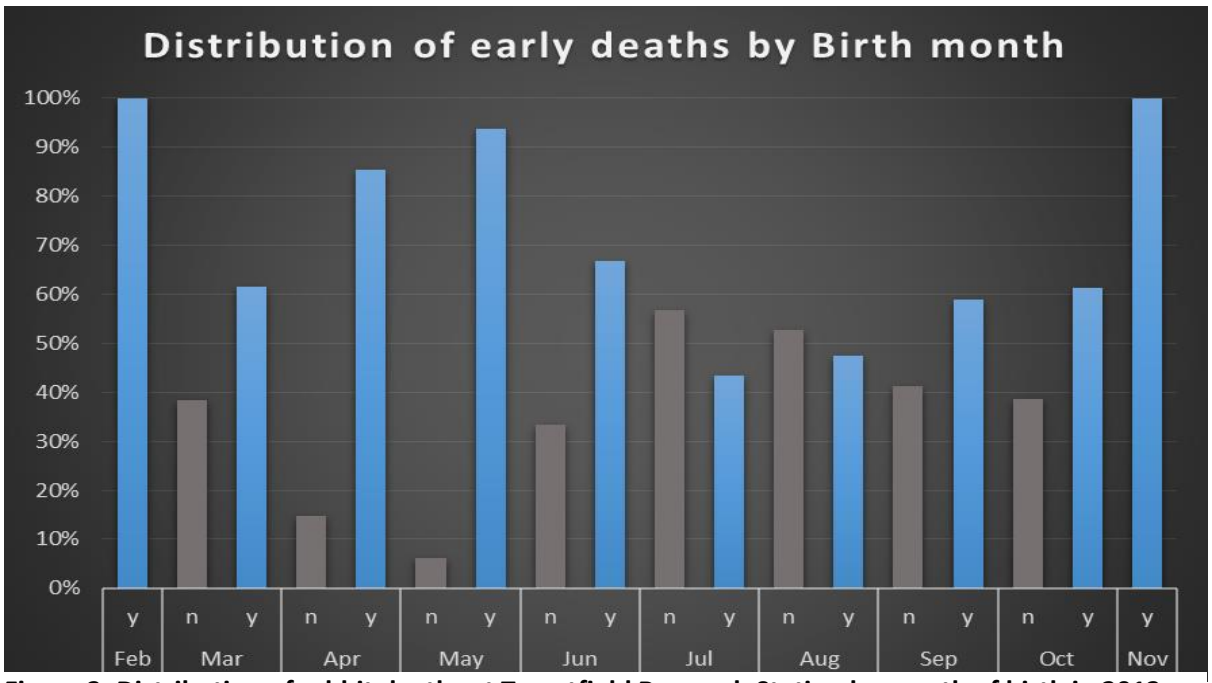

Figure 3: Distribution of rabbit deaths at Turretfield Research Station by month of birth in 2013 and 2014. Blue bars indicate proportion of rabbits dying before sexual maturity, grey bars indicate surviving offspring.

Directions for Further Study

The rich genetic dataset developed by this project, in conjunction with the Turretfield rabbit demographic and serological database, provides opportunity to examine the question of what drives variation in rabbit fitness in further depth, and also opens up new directions for investigation as outlined below.

All final findings will be submitted for publication in a peer reviewed international scientific journal during 2016, and forwarded to the Foundation for Rabbit Free Australia.

Genetic signatures

Preliminary analyses have revealed interesting patterns of heterozygosity and minor allele frequency spectrum within the rabbit population. These genetic signatures can be indicative of demographic history and the effect of selection on the genome, and warrant further investigation.

Further to this, many of the genetic loci of interest in genetic resistance to RHDV that were recently discovered by Nina Schwensow are likely to be present within this genetic database, given the (deliberate) similarity in sequencing methodology. As a result there is strong potential to analyse the variation in these loci within the population, and their correlation with survival and successful parentage.

Population structure

Principal component analysis of the SNP data obtained in this project places all of the Turretfield rabbits into a single cluster (see Figure 4 below), suggesting that it forms one discrete population, with slight genetic differentiation between geographically disjunct warrens – particularly the warren 9 complex which is located on a neighbouring hill to the other warrens. The presence of some outliers indicates a low level of potential immigration. The genetic structure of the Turretfield population, whether it conforms to the 'meta-population' ecological concept and the frequency of immigration, and frequency of inter-warren mating can be examined with greater clarity through the use of specific genetic analyses in the future.

Further potential effects on fitness

Using the serological database provided by BiosecuritySA we have the capacity to trace antibody titres for RHDV for the rabbits used in this study. In this way we can examine the impact of maternal antibody titre on offspring survival, as a measure of RHDV resistance conferred by the mother over the first few weeks of life.

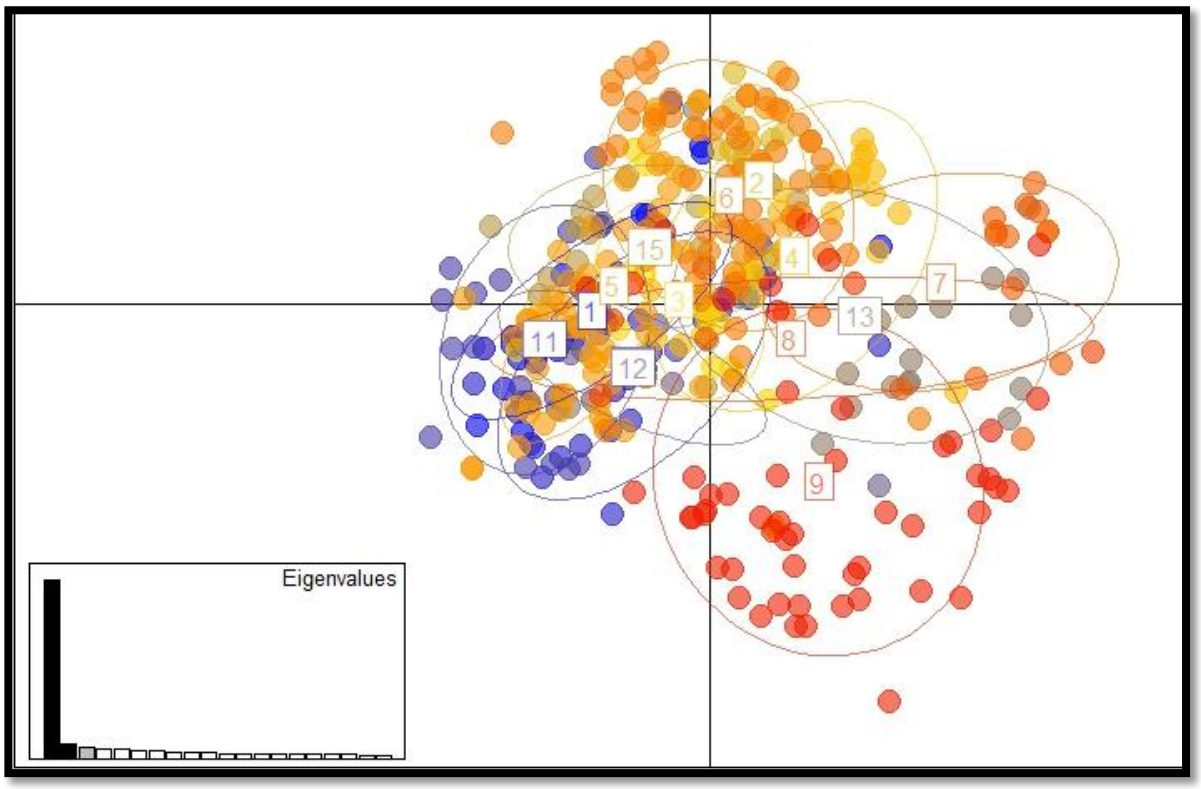

Figure 4: Principal Component Analysis of Turretfield SNP data, coloured by warren. Principal component 1 forms the x-axis (most explanatory component, shown in first bar of the eigenvalue plot), principal component 2 is on the y-axis and represents a considerably smaller portion of the variation.

Appendix: Study Methods and Materials

Study population

Turretfield Research Station, 50 km north of Adelaide, is the site of an on-going 17 year rabbit monitoring study. It contains around 17 warrens across a size gradient of approximately 1-40 holes (variable over time) and rabbits from this population have previously been shown to possess considerable resistance to RHDV infection (Elsworth 2012). Rabbits are trapped using wire treadle cage traps at 8-12 week intervals with sex, estimated date of birth, reproductive status and presence of clinical myxomatosis noted. Antibodies to RHDV and myxomatosis are tested using ELISA. The Turretfield rabbit database was made available for the purposes of this study, providing background demographic information.

During 2014 ear tissue samples for genetic analysis were taken from captured rabbits during regular monitoring at Turretfield and stored in 70% alcohol under University of Adelaide Animal Ethics Approval number S-2014-059. A total of 567 rabbits were sampled, which is expected to represent the vast majority of rabbits present over the year. Rabbits continued to be monitored throughout 2014 and 2015 to track the fate of sampled individuals, particularly with regards to the annual RHDV outbreak.

Extraction and sequencing

DNA was extracted from approximately 1/3rd of each ear tissue sample and then prepared in such a way that we obtained millions of short stretches of DNA from throughout the genome for each rabbit. The technical details of the procedure for this are below:

DNA was extracted from approximately 6mm^2 of each rabbit ear tissue sample using the Gentra Puregene tissue extraction protocol. Extract was used to prepare double digest GBS libraries

following the protocol of Poland 2012, using PstI as enzyme 1 and MspI as enzyme 2. Following the success of a pilot run, 96 samples including a negative control were pooled per run for sequencing on an Illumina NextSeq500 at the Australian Genome Research Facility with 75 cycle single-end reads in High output mode.

SNP determination

The sequences obtained for each rabbit were filtered for quality and to remove contaminants, and then matched to their corresponding locations of the rabbit genome. Fragments of DNA from the same place in the genome were then compared between rabbits to find genome locations that were variable between rabbits, called 'SNPs'. The collection of a rabbit's DNA at each of these SNPs becomes a kind of genetic fingerprint, inherited from the parents, which can be used to compare the rabbits. Again the technical process for this is below:

Raw sequence reads were filtered for quality (sliding window phred score limit of 10) and adapter presence, and demultiplexed using the process_radtags program from the software Stacks v1.34 (Catchen et al 2013). Reads were then mapped to the rabbit genome OryCun2.0 using the software Burrows-Wheeler Aligner (Li and Durbin 2009). The Stacks v1.34 pipeline ref_map was then used to call SNPs with default parameters. Files were checked for quality at each stage using FastQC. Yield was 81,140 SNP loci.

Pedigree construction

SNPs were further filtered to a subset of 7137 that were all successfully sequenced in at least 90% of the rabbits, displayed high variability (less frequent varieties still occurring at least 10% of the time) and were not located on sex chromosomes. From this set of SNPs 7 random subsets of 500 SNPs were generated as replicates for parentage testing – this was based on a power analysis by the software SOLOMON (Christie et al. 2013) which indicated that 150 SNPs would be sufficient to determine parentage with a vanishingly small chance of false pairing.

Rabbits were determined to be potential parents when aged at least 6 months older than a candidate offspring and known to be alive at the time of birth. The SNP subsets were used to search through the rabbits for genetically compatible parent-offspring combinations using likelihood methods as implemented by the software Cervus 3.07 using a simulation of 10,000 offspring, assuming 80% of the breeding population was sampled and requiring at least 150 comparable loci for a call to be made.

Consensus on a parent-offspring relationship was declared when the parent was identified with confidence in at least 4 out of 7 SNP subsets and the next most commonly identified parent (if any) was recognised by two or less SNP subsets. Pedigree Viewer v6.5f (Kinghorn 2015) was then used to display consensus relationships as a pedigree.

Of the 259 successfully sequenced offspring both parents could be identified with high confidence for 161 specimens, one parent for a further 54. Parentage could not be unambiguously determined for the remaining 44 rabbits. Much of the unresolved parentage is likely to be attributed to weak sequencing of particular parents or offspring. An analysis re-run using shortened sequence reads will be attempted to strengthen the sequence data for these individuals. Those with particularly poor sequencing outcomes will be re-sequenced in an additional future run, in order to resolve a greater majority of parent-offspring relationships.

The full pedigree image in Figure 5 below shows the success of this project in producing the intended rabbit pedigree, and just how complex the rabbit relationships are.

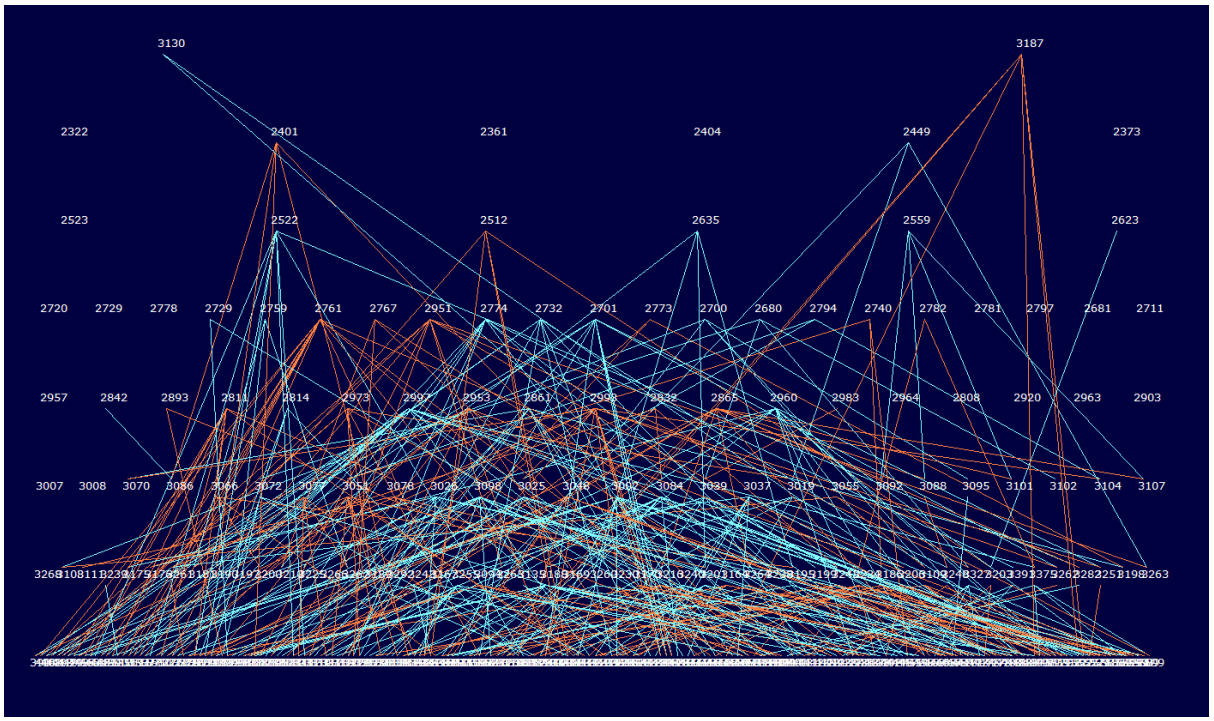

Figure 5: Pedigree of rabbits from Turretfield Research Station in 2013 and 2014. Paternal relationships indicated by blue lines, maternal relationships in orange, rabbit ID numbers in white. Vertical structure based on year of birth from 2005 (top) to 2014 (bottom row).

In order to better visualise the rabbit pedigree, the image can be restricted to rabbits meeting specific criteria such as rabbits from a specific warren (Warren 7 is shown in Figure 6 below), or those who survived until adulthood.

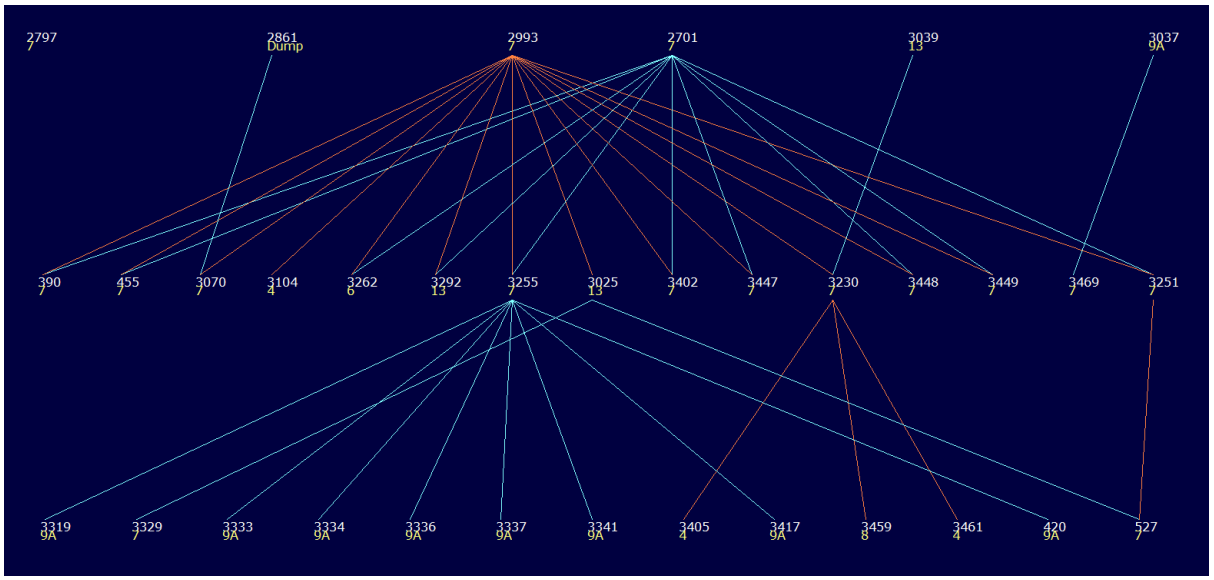

Figure 6: Pedigree of rabbits from Warren 7 at Turretfield and their immediate relatives. Paternal links in blue, maternal links in orange, rabbit ID numbers in white, warren of first known residence in yellow beneath. Note the dominant breeding male 2701 and female 2993. One of their male offspring, 3255 has migrated to Warren 9A and bred successfully in that warren.

Fitness and population structure

Population structure was examined using PCA, implemented in the R package adegenet, along with the distribution of missing data, heterozygosity and distribution of rabbits among warrens. Survival until breeding age was examined against warren of birth, sire, dam and month of birth using Microsoft Excel 2013's pivot table functionality.

Sample and data storage

The rabbit tissue collection continues to be stored in 70% alcohol, and extracted DNA in TE buffer. Both are intended to be archived at the University of Adelaide Biobank following completion of use. Sequence data will be deposited in secure long-term digital storage with eResearchSA.

References

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Molecular ecology*, *22*(11), 3124-3140.

Christie, M. R., Tennessen, J. A., & Blouin, M. S. (2013). Bayesian parentage analysis with systematic accountability of genotyping error, missing data and false matching. *Bioinformatics*, *29*(6), 725-732.

Elsworth, P. G., Kovaliski, J., & Cooke, B. D. (2012). Rabbit haemorrhagic disease: are Australian rabbits (Oryctolagus cuniculus) evolving resistance to infection with Czech CAPM 351 RHDV?. *Epidemiology and infection*, *140*(11), 1972-1981.

Kinghorn, B. & Kinghorn S. (2015) Pedigree Viewer v6.5f. Software hosted by the University of New England.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics*, *25*(14), 1754-1760.

Mutze, G., Bird, P., Jennings, S., Peacock, D., de Preu, N., Kovaliski, J., Cooke, B. & Capucci, L. (2015). Recovery of South Australian rabbit populations from the impact of rabbit haemorrhagic disease. *Wildlife Research*, *41*(7), 552-559.

Poland, J. A., Brown, P. J., Sorrells, M. E., & Jannink, J. L. (2012). Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. *PloS one*, *7*(2), e32253.