Final grant report form: Norman Hayward Fund

The terms and conditions of Animal Welfare Foundation Grants require recipients to complete a final report giving a summary of the work undertaken, including a layman's version which AWF can use in its own publications and in disseminating research results to the general public.

The final report is an opportunity for you to share details of the achievements and implications of the project that AWF has supported. The information is valuable as it allows AWF to assess and review the outputs and outcomes of the projects that we fund and enables the trustees to ensure that research has been carried out in accordance with AWF's charitable objectives.

Please complete and save this form electronically in Word and return it as an e-mail attachment to Erika Singh at erika@animalwelfarefoundation.org.uk. This is also the person you should contact if you have any queries about completing the report.

PROJECT/STUDY TITLE:	UNDERSTANDING THE ROLE OF STAT1, STAT3, AND STAT5 IN NATURALLY OCCURRING INFLAMMATORY LESIONS OF THE BOVINE AND OVINE MAMMARY GLAND.
PRINCIPAL APPLICANT(S)	Kate Hughes
GRANT AWARDED (DATE):	Awarded July 2016. Started December 2016.

Lay summary of project outcomes, achievements and potential impact on animal welfare: Max 300 words

Mastitis is painful inflammation of the udder that usually occurs as a result of bacterial infection. The premise of this project was to better understand the biology of ruminant udder cells in health and when responding to mastitis-causing bacteria. Signal Transducers and Activators of Transcription (STATs) are regulators controlling a plethora of vital mammary functions. We sought to delineate STAT functions in the ruminant udder, and in mastitis. Key findings are:

1. During udder development, STAT5 is expressed in duct epithelial cells and macrophages (immune cells), implying a role in immune surveillance and providing new insights into immune protection of the immature udder. Mastitis in heifers is particularly deleterious as it impacts udder health for the heifer's entire production lifespan.

2. We have demonstrated that mammary STAT3 is activated for a prolonged period around the day of birth. This is a time when ruminants are very susceptible to mastitis and we have shown that STAT3 upregulates other proteins that influence this susceptibility. In future, STAT3 or these upregulated proteins may be therapeutic targets which could be modulated to protect the udder at this vulnerable time.

3. STAT3 is activated in a subset of mastitis cases occurring during lactation. We have identified proteins regulated by STAT3 which likely influence pathogen survival and therefore constitute future therapeutic targets for mastitis treatment.

4. We examined supernumerary teats (additional teats) from ewes, initially to interrogate their STAT profile (as above). In doing so, we discovered two anatomically distinct groups of supernumerary teats. One group – anatomically complex – exhibited features suggesting that they are a potential risk factor for mastitis. We have therefore provided anatomical evidence to aid clinical decision-making surrounding teat removal. This will be helpful to veterinarians attempting to balance the welfare implications associated with removal with the mastitis risk of leaving supernumerary teats.

Detailed progress against original objectives: List outcomes against original objectives. Discuss what has been achieved, including any statistical analysis completed as part of the project.

The original aims of the project were threefold. Outcomes are therefore listed following each original project aim:

Aim 1: Definitively describe STAT1, STAT3 and STAT5 activation patterns during the ruminant mammary production cycle.

Key outcomes relating to aim 1:

- Phosphorylated STAT5 is expressed during postnatal development and appears to be associated with periods of allometric (as opposed to isometric) udder growth (pending final analysis currently in progress for manuscript). This finding provides new insights into immune protection of the immature udder. Mastitis in heifers is particularly deleterious as it impacts udder health for the heifer's entire production lifespan.
- STAT3 is activated by phosphorylation for a prolonged period around the day of birth in ruminants, in striking comparison to a very transient, and frequently overlooked, spike of activity on the day of birth in laboratory rodents. This is a very important finding as it has profound implications for understanding the ruminant mammary microenvironment around the day of birth and for unravelling the reasons why the gland may be susceptible to mastitis during this period. We have therefore interrogated this finding in detail. The window of pSTAT3 activation is from approximately 128 days gestation to at least 8 days lactation in sheep and from as early as 247 days gestation to at least 46 days lactation in cows. We have determined that mammary alveoli with any number of positive pSTAT3 epithelial cells are dramatically skewed towards either high or low numbers of luminal epithelial cells in which pSTAT3 is transcriptionally active. In other words, commitment to a STAT3 activity profile is usually at the level of the mammary alveolus (milk producing unit composed of multiple epithelial cells) rather than at the level of randomly scattered individual cells. This precise arrangement of cells in which pSTAT3 is active suggests an important function. Furthermore we have shown that pSTAT3 activity is not correlated with the degree of alveolar distension with colostrum secretion, which is important as it suggests it is not just a reflection of alveolar development. Critically, in both cows and sheep, alveoli expressing nuclear pSTAT3 in luminal epithelial cells are grouped with positive alveoli exhibiting significantly higher numbers of neighbouring alveoli that are also positive ($p<0.001$). Taken together, these findings suggest that STAT3 activity around the day of birth in ruminants has important implications for mastitis susceptibility.
- STAT3 is activated in a subset of mastitis cases occurring during lactation. Upregulation of STAT3 activity via phosphorylation is frequently associated with sub-acute and chronic infections. By contrast, STAT3 activity is less frequently seen in cases of gangrenous (necrotizing) mastitis in sheep and toxic mastitis in cows.

Aim 2: Identify the cellular compartment (epithelium, immune cells or stroma) which is expressing each STAT.

Key outcomes relating to aim 2:

- During udder development, STAT5 is expressed in duct epithelial cells and in lymphocytes and macrophages, implying a role in immune surveillance and providing new insights into immune protection of the immature udder.
- Around the day of birth, STAT3 is activated in ductal and alveolar mammary epithelial cells.
- In mastitis, STAT3 is activated in mammary epithelial cells surrounding the inflammatory focus, and in smaller numbers of macrophages, neutrophils, and fibroblasts.

Aim 3: Examine how the phenotype of the immune cell infiltrate is affected by the predominant STAT signalling axis.

Key outcomes relating to aim 3:

- We have identified a protein expressed by immunomodulatory macrophages that is regulated by STAT3 and likely influences pathogen survival.
- We have also identified a STAT3 target gene that is expressed by macrophages and mammary epithelial cells, the latter around the day of birth (see aim 1 above).
- Excitingly, both these proteins constitute possible future therapeutic targets for mastitis treatment.

Were there any challenges or barriers/modifications to the project? Explain the nature of and reasons for any changes in project focus, scope, delivery, schedule or evaluation.

There have been no significant changes in the project focus, delivery or evaluation.

Changes in scope:

1. The project was broadened slightly as a new collaboration with Prof Abby Fowden enabled us to culture primary mammary epithelial cells from sheep. This was very beneficial to the project as it enabled us to validate our results in a cell culture system (particularly regarding key findings 2 and 3 listed in the lay summary above). This helped to ensure that our data was even more compelling when subjected to scientific scrutiny and therefore has more potential to positively impact our understanding of ruminant udder health and welfare. A further benefit is that the knowledge gained from establishment of the cell culture system will be a lasting legacy of the project.
2. We employed a technique called CUBIC to optically clear the tissue and use deep three-dimensional imaging to visualize the udder in three dimensions. We are one of the first groups to have done this using bovine tissue and (to our knowledge) are the first group to do this in tissue from sheep.
3. We initially examined ovine supernumerary teats in order to interrogate their STAT profile. Whilst performing this analysis, we discovered that we could subgroup the supernumerary teats as simple or anatomically complex. Teats in the latter group have structures compatible with milk accumulation and thus likely constitute a mastitis risk. We developed these results into a scientific manuscript delineating the anatomy and microenvironment of supernumerary teats to provide anatomical evidence to aid clinical decision-making surrounding teat removal. We believe that this will be helpful to veterinarians attempting to balance the attendant welfare implications of supernumerary teat removal with the mastitis risk of leaving supernumerary teats in place. Since the presence of anatomically complex supernumerary teats may favour intramammary infection, we hope that this study will underpin further investigations and data-driven management decisions on the selection of replacement breeding animals without specific anatomical traits as a longer-term strategy that is preferable to surgical removal in young animals.

Challenges and changes in schedule:

1. As reported in our interim report, the STAT1 antibodies we trialled did not cross react reliably with ruminant tissue. However, we found that the STAT3 and STAT5 antibodies we were using were robust and reliable, so we focussed especially on the role of STAT3 and STAT5 in the ruminant mammary gland. This was particularly pertinent as we found that STAT3 was active around periods of gland susceptibility to mastitis and during mastitis events and is therefore the most exciting potential therapeutic target. Work on STAT1 during ruminant mammary gland development was largely replaced by expansion of the work examining the role of macrophages in mammary gland development to complement the discovery that STAT5 was active in macrophages during development.
2. A further challenge was difficulty in collecting sufficient bovine samples from different stages of the developmental cycle, which initially slowed progress on the bovine project particularly as co-applicant Dr Paul Wood moved to the University of Edinburgh soon after the project was established. Although sample collection is a drawback of using clinical samples, we feel strongly that the strength of using clinical samples is that they truly reflect the biology of the ruminant mammary gland and we believe this outweighs the drawback of challenging sample collection. This hurdle was overcome by the continued contributions of Dr Paul Wood from his new position, by forging additional new collaborations, and by the very generous understanding of the Norman Hayward Fund Trustees in granting us a no-cost extension to the project. We are extremely grateful to the Trustees for the no-cost extension which has allowed us to complete the project.

Provide details of knowledge transfer activities to date and any future plans/actions.

I have given 5 national and international research talks detailing the work directly resulting from this grant:

1. **Department of Biomedicine, University of Basel, Switzerland.** The role of STATs in the mammary microenvironment: A multi-species approach. October 2019.
2. **Ontario Small Ruminant Veterinary Conference, Guelph, Canada.** The multifaceted roles of STATs in ovine postnatal mammary gland development and mastitis. June 2019.
3. **Clinic for Ruminants, Ludwig Maximilian University of Munich, Germany.** Studying the udder in health and disease: A molecular pathology approach. March 2019.
4. **Sheep Veterinary Society Autumn Meeting, Windermere, UK.** Udder under the scope: New insights into the sheep mammary microenvironment in health and in mastitis. September 2018.
5. **International Sheep Veterinary Congress, Harrogate, UK.** Does STAT3 activity have a role in naturally occurring inflammatory lesions of the ovine mammary gland? May 2017.

In addition, I have given 3 local research talks detailing the work directly resulting this grant:

1. **Departmental Seminar, Department of Veterinary Medicine, University of Cambridge.** Udderly different? A comparative approach to studying the mammary gland in health and disease. February 2019.
2. **Shetland Seminar, Department of Veterinary Medicine, University of Cambridge.** Udder under the scope: The ruminant mammary microenvironment in health and disease. October 2018.
3. **Blitz talk, Research Afternoon, Departmental of Veterinary Medicine, University of Cambridge.** Unravelling STAT activity in the ovine mammary gland. July 2017.

The work directly supported by this grant has also been presented in the form of 9 poster presentations at international, national and local research conferences (one of which is forthcoming):

1. **Pathological Society Winter Meeting, London, UK.** Distribution of Ki67 expression, macrophages and pSTAT5 expression, during ruminant mammary postnatal terminal ductal lobular unit development: Insights for understanding postnatal breast development. Abstract accepted. Forthcoming January 2020.

2. **VPHA and AGV Autumn Meeting, Sequences and Consequences in Veterinary Public Health, Dunchurch, UK.** Macrophage distribution in prepubertal lamb mammary glands suggests an alternative function for mammary macrophages. October 2019.
3. **Research Afternoon, Departmental of Veterinary Medicine, University of Cambridge, UK.** Distinct mammary STAT3 activation dynamics are associated with parturition, involution, and a subset of mastitis cases in ruminants. July 2019.
4. **Mammary Gland Biology Gordon Research Conference. Newry, USA.** Distinct mammary STAT3 activation dynamics are associated with parturition, involution, and a subset of mastitis cases in ruminants. June 2019.
5. **Mammary Gland Biology Gordon Research Conference. Il Ciocco, Italy.** A multifaceted approach to delineating cellular interactions in mammary gland development and mastitis in sheep and rabbits. May 2018.
6. **Research Afternoon, Departmental of Veterinary Medicine, University of Cambridge, UK.** Is STAT3 a future therapeutic target in ovine and bovine mastitis? December 2017.
7. **British Cattle Veterinary Association. Southport, UK.** Does transcriptional activation of STAT3 have a role in ovine mastitis? October 2017.
8. **Research Afternoon, Departmental of Veterinary Medicine, University of Cambridge, UK.** Understanding the role of STAT transcription factors in the ovine mammary developmental cycle and in mastitis. July 2017.
9. **International Sheep Veterinary Congress. Harrogate, UK.** Does STAT3 activity have a role in naturally occurring inflammatory lesions of the ovine mammary gland? May 2017.

Provide details of any original peer-reviewed research papers, book chapters and books/monographs that have resulted directly from your work supported by this grant.

Peer reviewed research papers resulting directly from the work supported by this grant:

1. **Distribution of Ki67 expression, macrophages, and pSTAT5 expression, during ruminant mammary postnatal terminal ductal lobular unit development.** D Nagy, CMC Gillis, AL Fowden, J Wills, K Hughes. Manuscript in preparation for submission to Cell and Tissue Research.
2. **Distinct mammary STAT3 activation dynamics are associated with parturition, involution, and a subset of mastitis cases in ruminants.** LJA Hardwick, CJ Phythian, M Blanck, P Wood, AL Fowden, K Hughes. Manuscript in preparation for submission to Cell Death & Disease.
3. **Size of supernumerary teats in sheep correlates with complexity of the anatomy and microenvironment.** LJA Hardwick, CJ Phythian, AL Fowden, K Hughes. Revised manuscript currently under review - Journal of Anatomy.
4. **Pathology in Practice: Necrotizing (gangrenous) mastitis in a Lleyn cross ewe caused by *Staphylococcus aureus*.** O Greville-Heygate, B Reilly, K Hughes. Accepted manuscript in press - Journal of the American Veterinary Medical Association.
5. **The mammary microenvironment in mastitis in humans, dairy ruminants, rabbits and rodents: A One Health focus.** K Hughes, CJ Watson. Journal of Mammary Gland Biology and Neoplasia. 2018; 23 (1-2): 27-41. Review article. Full open access.
6. **The multifaceted role of STAT3 in mammary gland involution and breast cancer.** K Hughes, CJ Watson. International Journal of Molecular Sciences. 2018; 19 (6): 1695. Review article. Full open access.

Have any other funding bodies been involved in supporting the development of the work supported by this grant?

Not currently, but it is envisaged that the work supported by this grant will underpin imminent future (2020) grant applications to the Universities Federation for Animal Welfare (UFAW), the Biotechnology and Biological Sciences Research Council (BBSRC) and the National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3RS) for the continuation of this work, building on the findings to date.

Has any intellectual property activity has resulted directly from the research funded through this grant to date?

Not to date.

Have you, or any of the staff included above, received any prizes, awards or commendations as a direct result of the research supported by this grant to date? If yes please give details, including the name of the recipient.

Not to date.

If any clinical trials have been supported by the funding of this grant, please enter the title of the trial and briefly describe any key developments or outcomes (Max 300 words)

Not applicable.

Have the results been published? If yes please state when:

Not applicable.

Use the space below for any other relevant information you wish to report on.

Project legacies:

1. **Ruminant mammary tissue archive:** The project funding enabled examination of a large number of bovine and ovine mammary tissue samples from a panel of mammary postnatal developmental stages and from a range of cases of mastitis (many with accompanying microbiology results). Formalin fixed paraffin embedded tissues from these cases will form a permanent project legacy and will be an invaluable resource for future studies.
2. **Primary ovine mammary epithelial cells:** The project funding enabled creation of several primary ovine mammary epithelial cell lineages some of which remain frozen and will be an invaluable and key tool for future studies as a further project legacy.