



**An Roinn Talmhaíochta,
Bia agus Mara**
Department of Agriculture,
Food and the Marine

Research Stimulus Fund

Final Report

**'Molecular Biomarkers of Fertility - New Tools for the Cattle Industry,
'Reproductive Defensins'**

DAFM Project Reference No: 11/S/104

Start date: 01/01/2013

End Date: 31/12/2017

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Collaborating Research Institutions and Researchers:

Trinity College Dublin, Prof Cliona O' Farrelly
University College Dublin, Prof Pat Lonergan
University of Limerick, Dr Sean Fair

Please place one "x" below in the appropriate area on the research continuum where you feel this project fits

Basic/Fundamental	—————>	Applied	—————>	Pre Commercial		
1	2	3	4	5 X	6	7

Please specify priority area(s) of research this project relates to from the National Prioritisation Research Exercise* (NRPE) report;

Priority Area (s)	Sustainable Food Production and Processing
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Key words: Fertility, Defensins, Antimicrobial Peptides, Host defence

1. Rationale for Undertaking the Research

The Irish Cattle Breeders Federation (ICBF) and Animal Health Ireland (AHI) had previously identified infertility as a significantly detrimental issue for the agricultural Industry. Lost profit due to poor reproductive performance has been estimated at £100 (€118) annually per cow and with in-excess of 1.3 million dairy cows in Ireland, this represents a cost in excess of €153 million per year, which (even excluding the additional 1.3 million beef cows) is unsustainable. The decline in dairy cow fertility associated with intensive selection for production has been well documented, and is known to have a significant genetic component. However, fertility is also a major problem in the bull, and no single diagnostic test can accurately predict fertility in bulls producing apparently normal semen. Pregnancy rates are ~45-50% in dairy cows but can fall as low as 25% with low fertility bulls, and this variation is a major impediment to the use of high genetic merit bulls and limits genetic gain.

Therefore new tools to address the national infertility problem are critical and this proposal aimed to address two specific Industry relevant issues: 1. to determine how β -defensins mediate fertility in cattle and 2. to develop a genetic test that could also be incorporated into a selection index for improved fertility. With funding from a previous Research Stimulus call, we discovered novel β -defensin genes in cattle and when these genes are knocked out in mice (or mutated in human males) they dramatically affect male fertility. With the widespread use of AI (where low fertility bull sperm bypasses the cervix, and therefore mucus), the dissemination of genes associated with subfertility could very rapidly have a detrimental effect on the fertility of the national herd. We proposed that defensin haplotype of the bull will have a determining effect on fertility. Therefore, we proposed to identify which defensins are relevant to the fertility problem in cattle and how variation in these genes affects reproductive function.

2. Research Approach

This novel application sought to build a new platform in bovine reproductive genetics and immunology by linking the national leaders in these fields with strategic industry partners (NCBC) to address two key questions- 1. Is fertility mediated by defensin genotype and 2. Can defensin genotype predict fertility in cattle. Using our combined expertise, the mechanisms driving reproductive tissue-specific defensin expression will be uncovered - an important step toward identifying their epistatic roles in reproduction and immunity. A breed effect on fertility had been detected in sheep and our population genetics task aimed to uncover novel polymorphisms, and breed-specific frequency differences for incorporation into the Genomic Selection programme for improved fertility. Successful reproduction will depend on both parent's reproductive fitness and this proposal analysed the effect of defensin haplotype on bull fertility. Functional analysis aimed to identify the roles of these genes in sperm function, epithelial binding and the regulation of immunity in the female tract as well as in oocyte competence. Finally, this comprehensive dataset would help determine if these genes could form a useful diagnostic test and breeding target to identify cattle with superior fertility.

Research Methodologies employed:

- A. Targeted DNA sequencing of 19 novel reproductive β -defensin genes.
- B. Whole Exome-sequencing of all exons in the bovine genome between high and low fertility bulls. This was among the first uses of this technology in cattle.
- C. A gene-association study identified a significant association between these defensin genes and bull fertility.
- D. Population genetics and bioinformatics analysis of DNA sequence reads to identify polymorphic alleles that differed between cohorts. Bioinformatic tools were also employed to identify conserved genetic elements surrounding defensin genes across species to understand what stimuli are likely to drive their expression.
- E. Generation of novel bovine defensin antibodies, including against BBD126.
- F. Expression of recombinant BBD126 peptide for functional assays using *E. coli*.
- G. Development of a system to capture of the native BBD126 peptide from epididymal fluid.
- H. These important tools enabled us to describe the expression of defensins on bull sperm and in the male reproductive tract for the first time.
- I. Furthermore, functional analysis using bull sperm bovine oviductal cell cultures not only validated the association described above, but also enabled us to identify that BBD126 promotes sperm motility and binding to oviductal epithelium.
- J. In this way we identified novel candidate genes regulating bull fertility which we also validated and identified the underlying mechanism.
- K. SNPs identified during the course of the project, with bull fertility and beef cow fertility (WP8) have been added to the IDB SNP Chip for future association studies to refine other genes of relevance to agriculture as more accurate (and new) phenotypes become available.

Deviations from original proposal:

In addition to our original objectives, we managed to:

- Sequencing of an additional set of 44 genes which included other β -defensin and cathelicidin genes in high and low fertility bulls.
- Performed a whole-exome sequencing analysis in a subset of bulls which not only validated some of our findings using a different technology and approach but also identified a wealth of additional fertility-related genes.
- As part of the Government sponsored Jobsbridge programme, we recruited an individual who worked with the postdoctoral scientist employed on this grant to capitalise on the efforts described above and identify the equivalent genes in the sheep genome. This led to an additional publication.
- In collaboration with Ross University in St. Kitts, Rebecca Schleisman performed functional validation of these genes in the female tract. This was funded by the university.
- In collaboration with a number of partners, Sean Fair and Kieran Meade developed a stock bull fertility database which is a useful tool for the future evaluation of genes regulating stock bull fertility.

3. Research Achievements/Results

This was a highly productive project which led to multiple research achievements. These included sequencing the genetic diversity at these important genes in cattle for the first time. This enabled us to capture genetic variants present in the DNA from the Irish national herd, and consisted of both dairy and beef breeds. Using this database of variants, combined with fertility phenotypes on these bulls, we performed a number of scientific analyses (known as whole-exome sequencing and an association analysis), we identified genetic variants (known as a haplotype) associated with bull fertility. Therefore some bulls with this combination of genetic variants have higher fertility. This was the first evidence to link these genes to fertility in cattle. Furthermore, we then developed tools and techniques to enable us perform a detailed characterisation of how these genes contribute to fertility, and this was achieved by producing antibodies (to detect the protein that these genes code for) and adding them to sperm to analyse their function. By showing that addition of these proteins to sperm boosted sperm function (including their ability to bind to cells in the female reproductive tract), we could add biological meaning and reliability to our earlier findings. In addition, the genetic variants detected were also added to the SNP chip used nationally for cattle genotyping which may be of benefit to improved breeding for multiple agriculturally relevant traits as more phenotypes are collected in years to come. In summary, this study was the first to identify and characterise an unambiguous role for β -defensin genes in bull fertility. In answering Industry relevant questions, this project identified accurate diagnostic and breeding targets for improved fertility in cattle.

4. Impact of the Research

Industry: Fertility is critical to the sustainability of the livestock sector, particularly as one bull can potentially serve thousands of individual cows via artificial insemination. We cannot currently predict bull fertility *in vitro* accurately and as a result there is potential for any bull to pass on sub-optimal genes to the future generations. Industry has benefitted from an enhanced understanding of Irish AI bull genetics and the identification of genes which affect fertility as a result of this project. Furthermore, the NCBC have been involved in multiple additional projects with the project leader since this project ended.

End-users (farmers): Variants identified in these important genes have been added to the SNP chip used for national genotyping and as fertility and immunity in cattle will continue to be of commercial practical interest to farmers, the approaches developed herein will continue to contribute to future developments in agriculture for years to come. The model developed here can now also be exploited in other livestock species including sheep and pigs.

Scientific community: This project has brought together national and international leaders in the areas of reproduction, genetics and immunology to develop a deep understanding of the mechanisms underlying sperm function in bulls. From gene discovery to the discovery of an association with bull fertility concurrent with improved biological understanding means that this project has promoted new technologies, identified new scientific discoveries, advanced multi-disciplinary training in cutting edge science as well as fostering a greater collaborative relationship between academia and industry in a practical way. We now have a research pipeline for gene characterisation, enhanced in vitro assays to assess sperm function, a database of genetic mutations in genes which are not only important in fertility but also for immunity. This foundation has benefitted the generation of consortia for current and future scientific collaborations between the institutions. For example, we have already secured national funding from IRCSET for a PhD student to develop this work further and in collaboration with Dr Sean Fair, have secured EU-ERA-net funding for related work in sheep.

Other: Future proofing the industry against unforeseen consequences of the dissemination of genes associated with poorer fertility (or immunity) will be critical to maintaining farmer confidence in AI and supporting sustainable expansion of the cattle sector.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

- Collaborative links were developed with multiple universities including Ross University in St. Kitts
- Collaborations developed with industry including the National Cattle Breeding Centre and Irish cattle Breeding Federation

(ii) Outcomes where new products, technologies and processes were developed and/or adopted

- There is scope to add defensins to sperm straws to improve sperm function and this is being actively pursued via an application to Enterprise Ireland with NCBC.

(ii) Outcomes with economic potential

- SNPs discovered in these novel genes have been added to the IDB SNP chip which is an important resource for future genotyping and genome-wide association studies.
- These Defensins are host-defence peptides and future work is aiming to exploit their functions as natural alternatives to antibiotics. This has been submitted under the DAFM funding call 2019.

(iv) Outcomes with national/ policy/social/environmental potential

- A significant proportion of the Irish national herd are bred to stock bulls and the results described herein not only provide a useful template for follow on studies

but will enable rapid progress in the assessment of these genes in the stock bull population. Fertility is closely associated with immunity and ultimately these studies will contribute to breeding more efficient livestock.

4(b) Summary of Research Outputs

(i) Peer-reviewed publications, International Journal/Book chapters.

Fair S, Meade K, Reynaud K, Druart X, de Graaf SP. The biological mechanisms regulating sperm selection by the ovine cervix. *Reproduction*. 2019 Mar 1. pii: REP-18-0595.R1. doi: 10.1530/REP-18-0595. PMID: 30921769

Meade KG, O'Farrelly C. β -Defensins: Farming the Microbiome for Homeostasis and Health. *Front Immunol*. 2019 Jan 25;9:3072. doi: 10.3389/fimmu.2018.03072. PMID: 30761155

Fernandez-Fuertes B, Blanco-Fernandez A, Reid CJ, Meade KG, Fair S, Lonergan P. Removal of sialic acid from bull sperm decreases motility and mucus penetration ability but increases zona pellucida binding and polyspermic penetration in vitro. *Reproduction*. 2018 Jun;155(6):481-492

Lyons A, Narciandi F, Donnellan E, Romero-Aguirregomezcorta J, Farrelly CO, Lonergan P, Meade KG, Fair S. Recombinant β -defensin 126 promotes bull sperm binding to bovine oviductal epithelia. *Reprod Fertil Dev*. 2018 May 18

Fernandez-Fuertes B, Blanco-Fernandez A, Reid CJ, Fair S, Lonergan P. Removal of sperm sialic acid increases mucus penetration ability and zona pellucida binding but also polyspermic penetration in cattle. *Reproduction* 155(6):481-492. 2018.

Fernandez-Fuertes B, Rodriguez-Alonso B, Sanchez JM, Simintiras CA, Lonergan P, Rizos D. Looking at the big picture: understanding how the oviduct's dialogue with gametes and the embryo shapes reproductive success International Ruminant Reproduction Symposium, Foz do Iguacu, Brazil, September 2018. *Animal Reproduction* 15 (Suppl 1): 751-764.

Alkhodair K, Almhanna H, McGetrick J, Gedair S, Gallagher ME, Fernandez-Fuertes B, Tharmalingam T, Larsen PB, Fitzpatrick E, Lonergan P, Evans ACO, Carrington SD, Reid CJ. Siglec expression on the surface of human, bull and ram sperm. *Reproduction* 155(4):361-371. 2018.

Hall TJ, McQuillan C, Finlay EK, O'Farrelly C, Fair S, Meade KG. Comparative genomic identification and validation of β -defensin genes in the *Ovis aries* genome. *BMC Genomics*. 2017 Apr 4;18(1):27

Whiston R, Finlay EK, McCabe MS, Cormican P, Flynn P, Cromie A, Hansen PJ, Lyons A, Fair S, Lonergan P, O' Farrelly C, Meade KG. A dual targeted β -defensin and exome sequencing

approach to identify, validate and functionally characterise genes associated with bull fertility. *Sci Rep.* 2017 Sep 25;7(1):1228

Johnson GP, English AM, Cronin S, Hoey DA, Meade KG, Fair S. Genomic identification, expression profiling, and functional characterization of CatSper channels in the bovine. *Biol Reprod.* 2017 Aug 1;97(2):302-312

Fernandez-Fuertes B, Laguna R, Fernandez-Gonzalez R, Gutierrez-Adan A, Blanco-Fernandez A, Kelly AK, Kölle S, Lonergan P. Subfertility in bulls carrying a nonsense mutation in TMEM95 is due to failure to interact with the oocyte vestments. *Biology of Reproduction* 2017, 97(1): 50-60. PMID: 28859280.

Holden SA, Fernandez-Fuertes B, Murphy EM, Lonergan P, Fair S. Effect of seminal plasma from high and low fertility bulls on caudal epididymal spermatozoa function. *Reproduction Fertility and Development* 29: 2457-2465. 2017.

Holden SA, Fernandez-Fuertes B, Murphy C, Brennan L, Butler ST, Lonergan P, Fair S. Relationship between in vitro sperm functional assessment, seminal plasma composition and field fertility following AI with non-sorted or sex-sorted bull semen. *Theriogenology* 87:221-228. 2017.

Pasquariello R, Fernandez-Fuertes B, Strozzi F, Pizzi F, Mazza R, Lonergan P, Gandolfi F, Williams JL. Profiling microRNAs in bovine blastocysts using deep sequencing. *Reproduction Fertility and Development* 29: 1545-1555. 2017.

Fernandez-Fuertes B, Narciandi F, O'Farrelly C, Kelly AK, Fair S, Meade KG, Lonergan P. Cauda Epididymis-Specific Beta-Defensin 126 Promotes Sperm Motility but Not Fertilizing Ability in Cattle. *Biol Reprod.* 2016 Dec;95(6):122. Epub 2016 Oct 5.

Narciandi F, Fernandez-Fuertes B, Khairulzaman I, Jahns H, King D, Finlay EK, Mok KH, Fair S, Lonergan P, Farrelly CO, Meade KG. Sperm-Coating Beta-Defensin 126 Is a Dissociation-Resistant Dimer Produced by Epididymal Epithelium in the Bovine Reproductive Tract. *Biol Reprod.* 2016 Dec;95(6):121. Epub 2016 Oct 5

F Narciandi, A Lloyd, KG Meade, C O'Farrelly. A novel subclass of bovine β -defensins links reproduction and immunology. *Reprod Fertil Dev.* 2014;26(6):769-77. doi: 10.1071/RD13153. PMID: 23870162

Meade KG, Cormican P, Narciandi F, Lloyd A, O'Farrelly C. Bovine β -defensin gene family: opportunities to improve animal health? *Physiol Genomics.* 2014 Jan 1;46(1):17-28. doi: 10.1152/physiolgenomics.00085.2013. Epub 2013 Nov 12. PMID: 24220329

(ii) Popular non-scientific publications and abstracts presented at conferences

Abstracts presented at:

- AVTRW conferences over multiple years
- Agricultural Research Forum over multiple years (K. Meade was secretary)
- Innate defence mechanisms conference in the Netherlands
- Irish Society of Immunology annual meetings
- Gordon Research Conference in Italy
- International Society for Functional Genomics, Italy
- British Society for Animal Science, Edinburgh
- European embryo transfer meetings
- International embryo Transfer Society meetings

Popular/Outreach:

(iii) National Report

(iv) Workshops/seminars at which results were presented

-Male Fertility workshop in Teagasc in 2014 with international experts in attendance

(v) Intellectual Property applications/licences/patents

(vi) Other - Invited talks

Kieran Meade was invited to give the following talks:

- 'Ontogeny of intestinal immunity' at EU JPI meeting in UCD (November 2016).

- 'Distinguished lecturer' talk at the Fifteenth Annual Research Symposium of the University of Florida Graduate Program in Animal Molecular and Cell Biology 2017.

- Keynote address at 8th International Meeting on Antimicrobial Peptides (IMAP 2018),

- A summer student working with Sean Fair and Kieran Meade working on this project was the Altech global young scientist program winner presented in USA for Defensin research. See: <http://www.alltech.com/news/news-articles/2014/05/21/future-science-good-hands-alltech-young-scientist-winners-announced>.
- SNP discovery in bovine β -Defensin antimicrobial peptide genes. Invited talk given by Dr Emma Finlay at Roche Sequence Capture Symposium in Dublin Zoo (23/10/14).

Outreach: Our results were also disseminated widely at scientific outreach events including:

- Science week
- Discover Research Dublin
- Teagasc TResearch publications - "Genetics and bull fertility", Summer issue 2014. <https://www.teagasc.ie/publications/tresearch/> (P.36)
- UCD Observer newspaper - website undergoing maintenance
- Farming independent - <https://www.independent.ie/business/farming/beef/new-database-to-reveal-scale-of-infertility-in-stock-bulls-36209773.html>
- Postgraduate Research fora

5. Scientists trained by Project

Total Number of PhD theses: 3

Dr Anne Barry-Reidy, Regulation of Bovine β -defensin Expression (Awarded 2017, TCD)

Dr Ronan Whiston, Genetic Variation in Bulls Divergent for Fertility (Awarded 2017, TCD)

Dr Beatriz Fernandez, (Awarded 2017, UCD). Bea attended the highly prestigious and renowned Frontiers in Reproduction (FIR) course from 29/4/17-11/6/17 - an intensive 6-week-long laboratory and lecture course for scientists-in-training who seek to improve their knowledge and experimental skills in order to pursue a career in the Reproductive Sciences. FIR is held each summer at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts

Total Number of Masters theses: 2

Alan Lyons, The role of Bovine β -defensin 126 on the Ability of Bull Sperm to Bind to Oviductal Epithelium (Submitted August 2016 and subsequently awarded by UL).

Rebecca Schleisman, Characterization of beta-defensin gene expression in the genital tract of the ewe (submitted March 2019 to Ross University, St. Kitts). This MSc thesis project was based in sheep and therefore not directly relevant to the tasks in this project (which was focused on cattle). However Rebecca benefitted from the expertise developed during the course of this project and the extension of our work to sheep is an added output of relevance to the agricultural industry.

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
Teagasc	5	2.6
Trinity College Dublin	1	0.5
University College Dublin	2	0.8
University of Limerick	1	0.2
ICBF	1	0.3
Total	10	4.4

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates/Contract Researchers	4	7.531
PhD students	3	11
Masters students	1	1.080
Temporary researchers		
Other		
Total	8	19.611

8. Involvement in Agri Food Graduate Development Programme

NA

9. Project Expenditure

Total expenditure of the project: € 1,245,346.19

Total Award by DAFM: € 1,267,113.66

Other sources of funding including benefit in kind and/or cash contribution(specify): € 5,000

Breakdown of Total Expenditure

Category	Teagasc	TCD	UCD	UL	Total
Contract staff	324266.99				324,266.99
Temporary staff					
Post doctorates		87265.89			87,265.89
Post graduates	153833.22		65286.12	21721	240,840.34
Consumables	93817.44	66034.83	34586.20	10912.04	205,350.50
Travel and subsistence	19051.37	4189.62	7371.99	2542.82	33155.80
Sub total					
Durable equipment	52199.81				52,199.81
Other	46879.43			10099.37	10,099.37
Overheads	155314.80	47247.10	32173.29	10552.76	245,288
Total	845,363.15	204,737.44	139,417.60	55,827.99	1,245,346.19

10. Leveraging

Additional staff:

An MSc student was funded by Ross University, St. Kitts

A number of the project collaborators have gone on to secure additional research grants from various funding agencies on bull fertility.

Funding secured from Science Foundation Ireland (SFI), David Kenny, Sean Fair, Pat Lonergan

- SFI Investigators Programme (2017; €1.94 million). Title: An integrated multidisciplinary approach to revolutionise dairy cattle breeding, through the application of state-of-the-art technology to advance the identification, sexual maturation, fertility and availability of semen from genetically elite sires

Funding secured from Enterprise Ireland (EI) Sean Fair

- Commercialisation Fund (2014; €233,000). Title: Development of a Sperm Filtration Device

Funding secured from the Irish Research Council (IRC) Sean Fair and Kieran Meade

- Enterprise Partnership PhD Scheme. Student started in January 2018
- Postgraduate Scholarship Scheme PhD. Student started in October 2017
- Employment based PhD Scheme. Student started in February 2014.

- New Foundations Scheme. Three small projects funded over 3 years.
- Postgraduate Scholarship Scheme PhD. Student started in September 2013

Teagasc Walsh Fellowship PhD awarded to Kieran Meade and Cliona O' Farrelly to investigate the role of these genes in the immune response to viruses. Student started in 2017. Project runs until 2021.

11. Future Strategies

The results from this DAFM funded project have laid the foundation for the researchers to secure additional competitive funding and building critical mass in the area of bull fertility research in Ireland. The researchers have secured follow on grants from SFI, EI, DAFM and the IRC to work on both basic and applied themes. All of these grants have significant input from the Irish cattle breeding companies. The researchers have broadened their collaborations and are now working with a multi-disciplinary team of world renowned scientists from agri-food, basic biochemistry and mathematical backgrounds together with key industry stakeholders in order to address bull fertility problems limiting the potential growth of Ireland's agri-food industry. Indeed, these collaborations have led to a recent H2020 funding application on the epigenetic regulation of bull fertility.

Taken together, the newly funded projects will characterise DNA and other biochemical markers of early puberty as well as unravelling the metabolic control of this complex trait so that it can be consistently advanced, through strategic early life dietary management. In addition, through the application of state-of-the-art genetic, physiological, immunological and molecular approaches, combined with *in vitro* sperm functional and bioinformatics analyses we aim to develop biomarkers to predict and monitor semen fertility. In parallel to this, the use of both liquid and sex sorted semen in the dairy sector is being further developed and trialled under Irish conditions. This novel, multidisciplinary and industry focused work will revolutionise cattle breeding in Ireland through augmenting the current genomic selection based cattle breeding programme and will further enhance the countrys' reputation as a world leader in the application of genomic technology to livestock breeding.

Kieran has submitted a new application under the DAFM funding call in 2019 which develops a number of themes started under the current grant application. The focus is on the exploitation of these molecules as alternatives to antibiotics with one avenue to include them in semen straws to reduce our reliance on exogenous antibiotics.

In addition, we are developing an application for a Marie-Curie International Training Network (ITN) to combine our nationally funded efforts with other EU funded consortia across the EU.