final report

Project code: B.AWW.0194

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Date published: May 2013

PUBLISHED BY
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NORTH SYDNEY NSW 2059

GonaCon™ trial in heifers

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

The project investigated the utility of the immunocontraceptive vaccine GonaCon™ to induce antibodies against the key reproductive hormone gonadotrophin releasing hormone (GnRH) in heifers, and suppress ovarian activity long-term. Heifers undergoing normal ovarian cycles received either a single GonaCon™ vaccination (n = 10) or double GonaCon™ vaccination (n = 9). The interval between primary and secondary vaccination for the double vaccination group was 60 days. Single vaccination was associated with uniformly low anti-GnRH antibody titres and ovarian activity was suppressed in 1/10 heifers. Double vaccination induced significant anti-GnRH antibody titres in 6/9 heifers after secondary vaccination, and ovarian activity was suppressed for 330 days in 5/9 heifers. Heifers subjected to single vaccination received 3 mg GonaCon™ and heifers subjected to double vaccination received 2 mg GonaCon™ (primary vaccination) and 1 mg GonaCon™ (secondary vaccination). The doses of GonaCon™ utilised could have been at the margin for cattle and the recommendation from the project was that higher doses of GonaCon™ (potentially 5 mg and 3 mg, respectively, for primary and secondary vaccination) should be investigated in heifers. Also, double vaccination with GonaCon™ would seem to be required to induce significant anti-GnRH antibody titres, and an immunocontraceptive response, in cattle in northern Australia.

Executive summary

The nature of beef production in northern Australia often makes it difficult to keep different classes of cattle (e.g. breeding and non-breeding heifers and cows) as separate herds and it is also difficult to tightly control the dispersal of bulls, both within and between properties. This creates problems with the prevention of pregnancies in heifers and cows that are surplus to breeding requirements and are deemed 'turn-off' animals. The solution has been surgical intervention (spaying) to prevent pregnancies and it is estimated that 400,000 to 500,000 animals, or potentially more, are spayed each year in northern Australia. All spaying techniques are invasive and can have unintended production and welfare outcomes. Accordingly, the beef industry is seeking to identify and introduce practical and cost effective technology to replace spaying.

Gonadotrophin releasing hormone (GnRH) has a fundamental role in reproduction both in females and males and the neutralisation of GnRH in blood has received considerable attention as an alternative to spaying and castration.

This project investigated the utility of the immunocontraceptive vaccine GonaCon™ to induce neutralising anti-GnRH antibodies in heifers and thereby suppress ovarian activity long-term. It was found that double vaccination with GnRH was required to induce significant anti-GnRH antibody titres and achieve long-term suppression of ovarian activity (330 days and potentially longer) in heifers.

The GonaCon™ vaccine incorporates attenuated *Mycobacterium avium* (*M. avium*) cell wall components which is important in generating an immune response. *Mycobacterium avium* and closely related organisms are endemic in many regions globally and the GonaCon™ vaccine relies on pre-expose to *M. avium* and/or related organisms so that immune memory is evoked when individuals are vaccinated with GonaCon™. The ensuing immune response to *M. avium* is accompanied by an immune response to a GnRH conjugate also in the vaccine, which results in the generation of anti-GnRH antibodies. This rationale for the formulation of GonaCon™ has resulted in strong immunocontraceptive responses in wildlife, domestic and production animals, primarily in North America but also in southern Australia (Canberra) in kangaroos, after single vaccination with GonaCon™. *Mycobacterium avium paratuberculosis* is the causative agent for Johne's Disease which occurs in southern Australia and the failure of heifers in southern Queensland to show a response to single vaccination with GonaCon™ confirmed that the Johne's organism is not prevalent in northern Australia.

The response to double vaccination with GonaCon™ in heifers was not consistent and 6/9 heifers had significant anti-GnRH antibody titres after secondary vaccination and 5/9 showed long-term suppression of ovarian activity. It was concluded that the doses of GonaCon™ used for primary and secondary vaccination were likely marginal for a consistent and repeatable response in cattle.

Therefore, the recommendation from the project is that higher doses of GonaCon™ are investigated. GonaCon™ should be further investigated in cattle given that the evidence from the project is that a long-term immunocontraceptive response can be evoked in cattle if significant anti-GnRH antibodies are induced.
Also, the neutralisation of GnRH with a vaccine is not a gender-specific approach and the same technology would have application in both female and male cattle.

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1. Background

Beef production in northern Australia occurs in extensive production systems and the typical infrastructure of these enterprises often makes it difficult to keep different classes of cattle (e.g. breeding and non-breeding heifers and cows) as separate herds. It is also difficult to tightly control the dispersal of bulls, both within and between properties. This creates problems with the management of groups of heifers and cows that are surplus to breeding requirements and are deemed 'turn-off' animals. It is often necessary to retain turn-off females for periods of 12 to 18 months in order to achieve optimal sale value. It is during this period that turn-off heifers and cows can conceive which impacts on production and animal management within enterprises. Surgical intervention (spaying) is currently used to prevent pregnancy in turn-off heifers and cows. It is estimated that 400,000 to 500,000 animals, or potentially more, are spayed each year in northern Australia (MLA pers. comm.).

Reproductive function in females relies on gonadotrophin releasing hormone (GnRH) which is released from the base of the brain and is transported by a blood capillary network to gonadotrope cells in the anterior pituitary gland where it stimulates release of the gonadotrophic hormones, luteinising hormone (LH) and follicle stimulating hormone (FSH). Both LH and FSH are required for normal functioning of the ovaries.

Given the fundamental role of GnRH in reproduction, the interception of GnRH before it can act at the pituitary gland has received considerable attention as an alternative to spaying and also castration in cattle.

The most common approach to neutralising GnRH has been to induce animals to generate antibodies to GnRH (13, 30). These antibodies circulate in blood and bind GnRH with high affinity. This prevents GnRH from binding to receptors on gonadotrope cells to stimulate the synthesis and release of LH and FSH. Whilst research on GnRH vaccines in cattle has been ongoing for about 25 years, immunisation technology has yet to be developed which achieves a sustained suppression of reproductive function in either bulls or cows. Technology is particularly lacking which maintains anti-GnRH antibodies long-term above threshold levels required to bind and neutralise the majority of GnRH in blood. This can be explained, in part, by the focus on GnRH vaccines that are required to be effective for relatively short periods in feedlot (1-2, 4-5, 10-12, 16, 21, 32-33, 42-43) and pasture-based (3, 8-9, 14, 17-19, 34-37, 40) production systems. The duration of effective immunocontraception after vaccination of cattle against GnRH using current technology is around 26 to 30 weeks (10, 14, 19-20, 41) and this typically requires multiple vaccinations to maintain adequate anti-GnRH antibody titres (41). A GnRH vaccine that aimed to achieve a longer-term immunospayed response in beef production systems in northern Australia did not consistently induce the required profile of anti-GnRH antibodies in sufficient numbers of heifers and cows (15, 20). A relatively small proportion of bulls immunised with the latter vaccine showed sustained testicular atrophy but the basis of this response in some individuals was not investigated (6).

Notwithstanding the limitations of current GnRH vaccine technology, commercial cattle vaccines are available which use conventional chemistry (18-19, 40) (Bopriva ®, http://www.zoetis.com.au/documents/e/ 1744/8042,2011%20MSDS%20Bopriva.pdf) and bacterial expression systems (10-11, 39) (Repro-BLOC, http:// ampliconexpress.com/vaccine/technology.html).

The incentive to develop longer-acting GnRH vaccines has actually been driven by the need to control native and introduced wildlife (7, 22, 24-25, 27, 31, 43), homeless cats and dogs (23, 29) and feral animals (26-27). This has led to the development of GonaCon™ which has been shown to suppress reproductive function for periods of 3 to 5 years including in ruminants (22, 24-25). A feature of GonaCon™ is that it can induce longer-term immunocontraception with a single vaccination.

GonaCon™ has achieved longer-term infertility after single vaccination because it contains a key component, *Mycobacterium avium* (*M. avium*). *M. avium* and related mycobacteria are considered to be endemic throughout the world and cause exposure in wildlife, livestock and domestic animals. GonaCon™ also contains GnRH conjugated to an immunogenic carrier protein. Hence, the rationale for GonaCon™ is that vaccination induces recognition by the immune memory system of *M. avium* and/or related organisms, and the ensuing immune response includes the generation of anti-GnRH antibodies. *M. avium paratuberculosis (MAP)* is the causative agent of Johne's Disease which occurs in southern Australia where it is thought to persist in livestock and wildlife. Evidence for the latter was provided by the long-term immunocastration response of kangaroos in Canberra to single vaccination with GonaCon™ (L. Hinds, personal communication).

Based on the published literature on longer-term immunocontraception achieved with GonaCon™ across a range of species, and the findings in kangaroos in southern Australia, GonaCon™ was evaluated for its efficacy in suppressing ovarian activity in heifers in northern Australia. Single vaccination with GonaCon™ did not induce significant anti-GnRH antibodies in heifers as had been anticipated (MJ D'Occhio and LA Miller, unpublished). This was subsequently explained, at least in part, by the lack of any apparent titres to *M. avium* or related organisms at the time of vaccination with GonaCon™ (LA Miller and MJ D'Occhio, unpublished). It was concluded from these findings that *M. avium* and closely related organisms are not endemic in northern Australia and the lack of prior exposure would explain why heifers did not show a response to single vaccination with GonaCon™. In support of this conclusion, Johne's Disease is not prevalent in northern Australia, although there have been some recent incidences.

If the above conclusion was correct, then it could be predicted that heifers and cows in northern Australia would respond to two vaccinations with GonaCon™ and that an immunospayed response would be maintained long-term.

The aim in this project, therefore, was to determine the response of heifers to either single or double vaccination with GonaCon™ and to ascertain the duration of any immunospayed response induced by GonaCon™.

2. Project objectives

The project objectives were:

1. Measured and reported on the suppression of ovarian function in beef heifers through the use of a single and double vaccination with GonaCon.

2. Measured and reported on *Mycobacterium paratuberculosis* (MAP) antibody titres and anti-GnRH antibody titres in heifers after single and double vaccination with GonaCon.

3. Methodology

Approvals

Animal ethics

The project was approved by the Production and Companion Animal, Animal Ethics Committee (PCA AEC) of the Animal Welfare Unit, The University of Queensland (Permit SAS/383/11/MLA).

Australian Pesticides and Veterinary Medicines Authority (APVMA)

The project was approved by APVMA (Permit 13015).

Australian Quarantine and Inspection Service (AQIS)

The project was approved by AQIS (Permit IP10005628).

Animals

High grade Brahman (Zebu, *Bos indicus*) heifers between 2 and 3 years of age were used in the Project. All heifers were showing regular ovarian cycles at the start of the project.

The heifers were randomly assigned on live weight to one of three treatments:

(i) Control heifers that were vaccinated with sham GonaCon™ that contained all components except the GnRH-carrier protein conjugate (n = 5);

(ii) Single vaccination with GonaCon™ (3.0 mg) (n = 10); and

(iii) Double vaccination with GonaCon™ at an interval of 60 days between primary (2.0 mg GonaCon™) and secondary (1 mg GonaCon™) vaccination (n =10).

The heifers were maintained on pasture and standard cattle management. They received supplementary feed of lucerne-hay as required in order to maintain a progressive increase in live weight towards a mature adult size and body condition of 3.0 to 3.5 (Scale 1.0 to 5.0).

GonaCon™ vaccine

The formulation of GonaCon™ is given in Appendix 1.

Vaccination was by intramuscular injection on the dorsal surface of the neck and immediately behind the head.

Anti-GnRH antibody titres

Anti-GnRH antibody titres were determined by ELISA as previously described (22-27).

Mycobacterium paratuberculosis* (MAP) *titres

Mycobacterium paratuberculosis (MAP) titres were determined by ELISA as previously described (22-27).

Ovarian follicular status

Ovarian follicular status was determined by trans-rectal ultrasonography using an Aloka 500 Echo Camera equipped with a 7.5 MHz transducer. Ovarian status was scored in the following categories (Appendix 2):

1. Score 1: suppressed follicular growth at early antral status (≤ 5 mm diameter for Brahman (Zebu, *Bos indicus*);

2. Score 2: follicular growth without ovulation (6 mm to > 10 mm diameter; follicles can grow to a relatively large size and not ovulate); and

3. Score 3: follicular growth with ovulation and corpus luteum (cows that show ovulation and have a corpus luteum are considered cyclic or oestrus)

4. Results

Live weight

Results for changes in live weight are shown in Tables 1 to 3. Heifers showed a progressive increase in live weight and there were no apparent differences in live weight gain between control heifers and heifers vaccinated with GonaCon™.

Mycobacterium paratuberculosis (MAP) *titre*

Results for *Mycobacterium paratuberculosis* (MAP) titres are shown in Tables 4 to 6 and Figures 1 to 3. MAP titres were not significant on Day 0 (start of the project) but there was a significant ($P < 0.0001$) increase in MAP titres from Day 0 to Day 60 for all three groups of heifers. There were no differences ($P > 0.05$) in MAP titres between the three groups on Day 60. Control heifers and heifers that received a single GonaCon™ vaccination tended to show a decrease in MAP titres from Day 60

to Day 89. Heifers that received double GonaCon™ vaccination tended to show an increase in MAP titres from Day 60 to Day 89 but this was not significant (P = 0.74).

Table 1. Changes in live weight for individual control heifers.

Heifer	Live weight							
	Day of study							
	0 Dec 2011	60 Feb 2012	89 Mar 2012	126 Apr 2012	154 May 2012	209 Jul 2012	316 Nov 2012	391 Jan 2013
11	498	542	570	588	584	606	630	692
12	456	490	522	562	564	558	592	614
17	398	430	456	476	484	492	518	550
21	390	412	424	444	458	444	464	512
26	446	482	508	530	546	542	578	606

Table 2. Changes in live weight for individual heifers that received a single GonaCon™ vaccination on Day 0.

Heifer	Live weight							
	Day of study							
	0 Dec 2011	60 Feb 2012	89 Mar 2012	126 Apr 2012	154 May 2012	209 Jul 2012	316 Nov 2012	391 Jan 2013
1	420	432	458	466	470	478		
3	412	430	454	480	512	490		
4	482	528	574	588	604	596	630	674
7	430	450	480	510	526	530		
8	460	480	520	552	562	546		
9	452	482	522	548	566	556		
19	420	456	484	508	514	516		
23	432	472	498	522	526	536		
24	406	434	460	486	496	496		
27	378	406	444	470	480	476		

Table 3. Changes in live weight for individual heifers that received a double GonaCon™ vaccination on Day 0 and Day 60.

Heifer	Live weight							
	Day of study							
	0 Dec 2011	60 Feb 2012	89 Mar 2012	126 Apr 2012	154 May 2012	209 Jul 2012	316 Nov 2012	391 Jan 2013
5	458	512	552	602	626	618	644	690
6	418	430	424	448	458	466	510	550
10	416	448	482	508	518	528		
13	386	410	434	452	454	436		

14	438	480	498	530	542	534		
16	482	512	544	572	592	590	644	670
18	450	484	504	542	560	552		
20	426	460	494	524	532	546	574	622
22	398	418	446	482	476	474	492	530
25	424	440	458	486	500	494		

Table 4. *Mycobacterium paratuberculosis* (MAP) titres for individual control heifers.

Heifer	***Mycobacterium paratuberculosis* titre**		
	Day of study		
	0 **Dec 2011**	**60** **Feb 2012**	**89** **Mar 2012**
11	0.440	1.808	1.483
12	0.054	1.321	0.997
17	0.020	0.935	0.939
21	0.322	0.846	0.850
26	0.038	1.050	0.645
mean	0.175	1.192	0.098

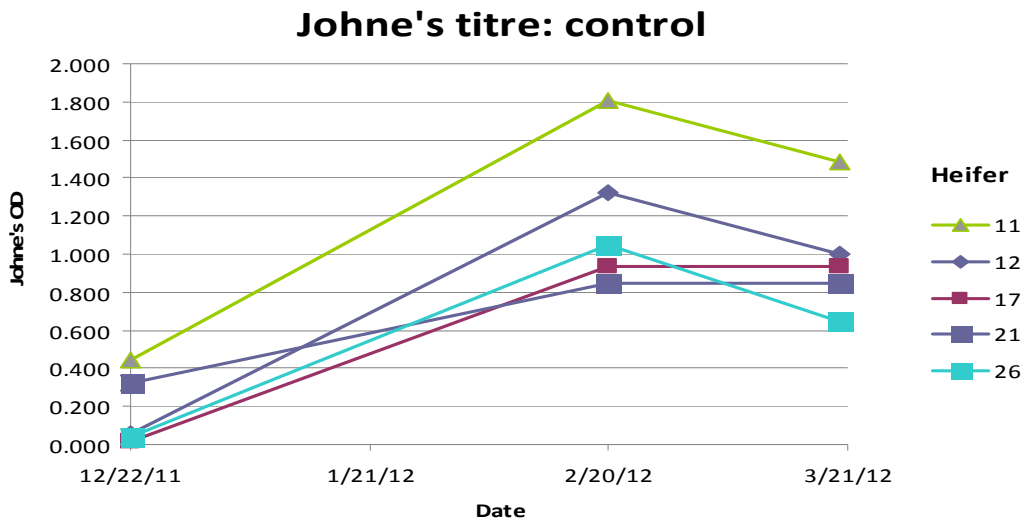

Figure 1. Longitudinal changes in *Mycobacterium paratuberculosis* (Johne's) titres for individual control heifers on Days 0, 60 and 89.

Table 5. *Mycobacterium paratuberculosis* (MAP) titres for individual heifers that received a single GonaCon™ vaccination on Day 0.

Heifer	***Mycobacterium paratuberculosis* titre**		
	Day of Study		
	0 **Dec 2011**	**60** **Feb 2012**	**89** **Mar 2012**
1	0.100	0.183	0.171
3	0.019	0.356	0.158
4	0.174	0.321	0.201
7	0.141	1.316	1.234
8	0.112	0.893	0.649
9	0.147	0.097	0.080
19	0.056	0.214	0.133
23	0.044	1.054	0.901
24	0.072	0.701	0.368
27	0.283	1.126	0.868
mean	0.115	0.626	1.164

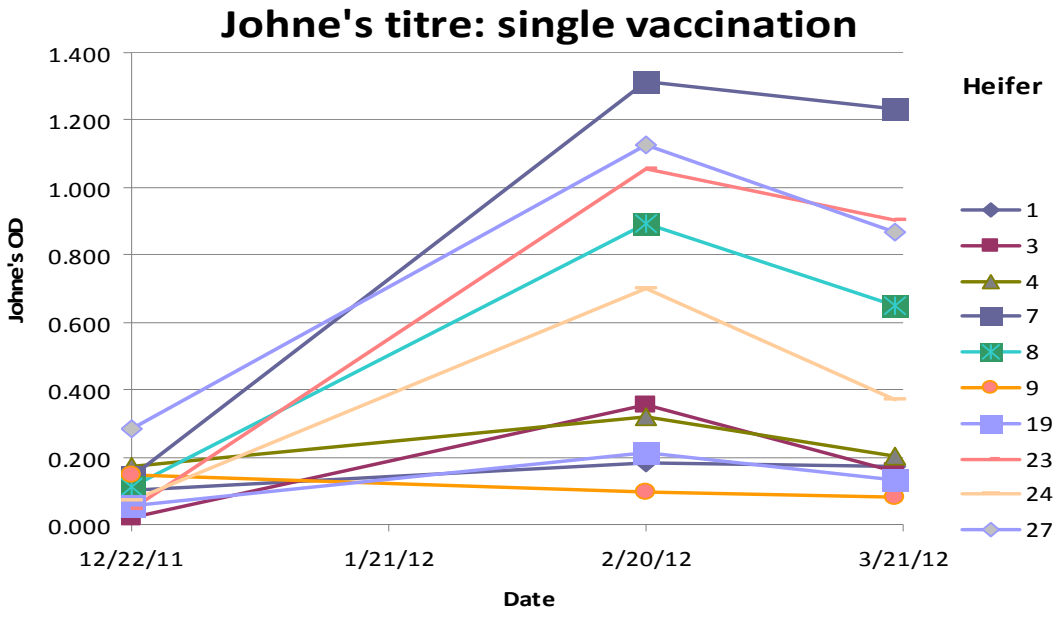

Figure 2. Longitudinal changes in *Mycobacterium paratuberculosis* (Johne's) titres on Days 0, 60 and 89 for individual heifers that received a single GonaCon™ vaccination on Day 0 (12/22/11).

Table 6. *Mycobacterium paratuberculosis* (MAP) titres for individual heifers that received a double GonaCon™ vaccination on Day 0 and Day 60.

Heifer	***Mycobacterium paratuberculosis* titre**		
	Day of Study		
	0 **Dec 2011**	**60** **Feb 2012**	**89** **Mar 2012**
5	0.147	1.545	1.841
6	0.047	0.661	1.192
10	0.023	0.091	0.761
13	0.085	2.023	2.482
14	0.365	1.238	1.641
16	0.084	0.905	0.678
18	0.022	0.043	0.452
20	0.027	0.025	0.097
22	0.246	1.058	2.287
25	0.043	0.034	0.205
mean	0.109	0.762	1.164

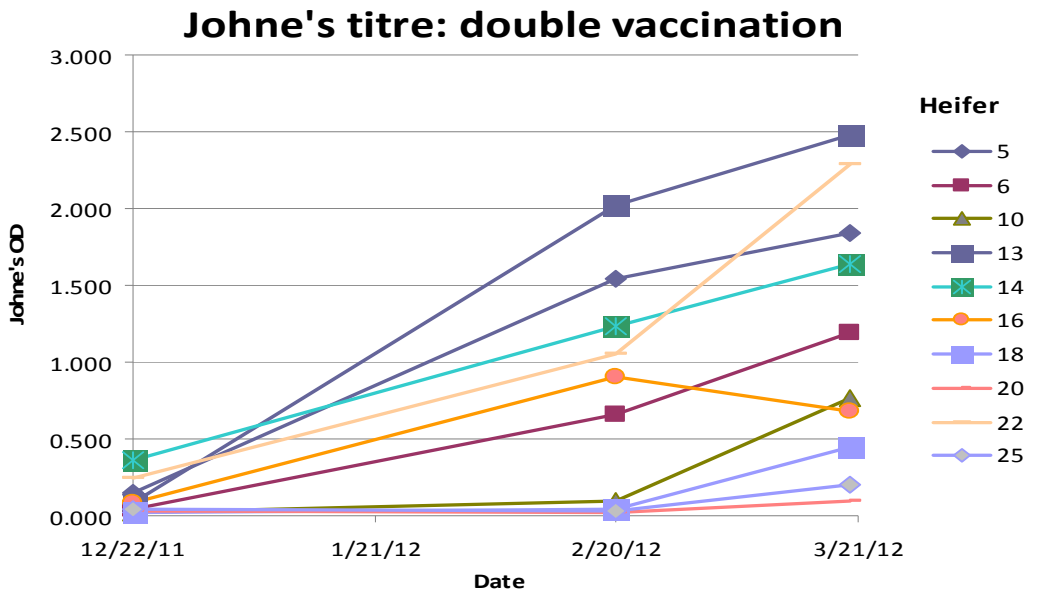

Figure 3. Longitudinal changes in *Mycobacterium paratuberculosis* (Johne's) titres on Days 0, 60 and 89 for individual heifers that received a double GonaCon™ vaccination on Day 0 (12/22/11) and Day 60 (2/20/12).

Anti-GnRH antibody titres

Results for anti-GnRH antibody titres are shown in Tables 7 to 9 and Figures 4 to 6.

Control heifers did not have anti-GnRH antibody titres (Table 7, Figure 4). Heifers that received a single GonaCon™ vaccination showed very modest anti-GnRH antibody titres at 60 days after vaccination and titres had declined by 89 days after vaccination (Table 8, Figure 5). Heifers that received a double GonaCon™ vaccination also had modest anti-GnRH antibody titres at 60 days after primary vaccination, except for Heifer 6 (Table 9, Figure 6). A secondary vaccination at Day 60 induced significant titres in 6/9 heifers (Heifers 5, 6, 13, 18, 20, 22) and titres were lower for Heifers 10, 16 and 25 (Table 9, Figure 6). Heifer 14 was found retrospectively not to have received a primary vaccination and nine heifers in this group are considered for the project report.

Table 7. Anti-GnRH antibody titres for individual control heifers.

Heifer	Anti-GnRH antibody titre		
	Day of Study		
	0 Dec 2011	60 Feb 2012	89 Mar 2012
11	na	0	0
12	na	0	0
17	na	0	0
21	na	0	0
26	na	0	0

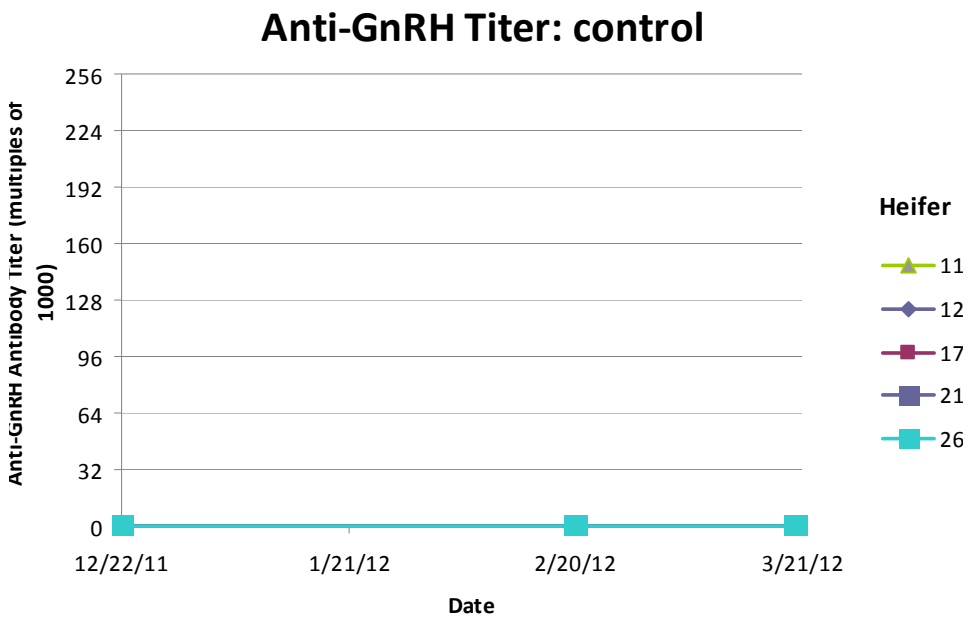

Figure 4. Longitudinal changes in anti-GnRH antibody titres in control heifers.

Table 8. Anti-GnRH antibody titres ($x10^{-3}$) for individual heifers that received a single GonaCon™ vaccination on Day 0.

Heifer	Anti-GnRH antibody titre		
	Day of Study		
	0 Dec 2011	60 Feb 2012	89 Mar 2012
1	na	8	1
3	na	8	1
4	na	1	1
7	na	2	1
8	na	8	1
9	na	0	0
19	na	0	0
23	na	2	na
24	na	0	1
27	na	8	2

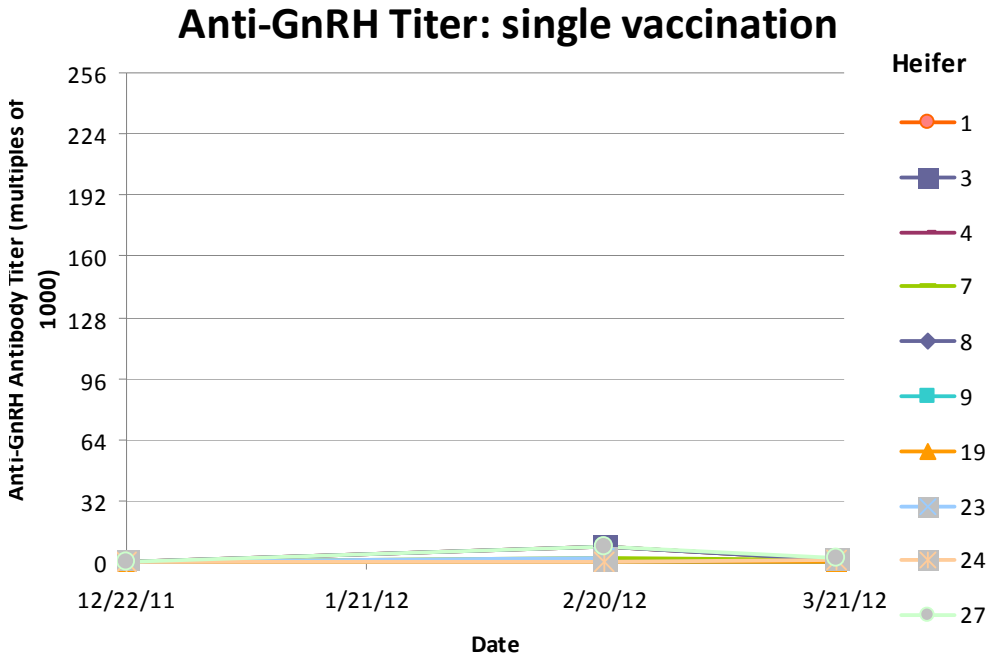

Figure 5. Longitudinal changes in anti-GnRH antibody titres in heifers that received a single GonaCon™ vaccination on Day 0 (12/22/11).

Table 9. Anti-GnRH antibody titres ($x10^{-3}$) for individual heifers that received a double GonaCon™ vaccination on Day 0 and Day 60.

Heifer	Anti-GnRH antibody titre		
	Day of Study		
	0 Dec 2011	60 Feb 2012	89 Mar 2012
5	na	1	> 128
6	na	128	> 128
10	na	1	16
13	na	1	> 128
14 †	na	0	0
16	na	4	32
18	na	0	> 128
20	na	8	> 128
22	na	0	128
25	na	1	8

† heifer did not receive a primary vaccination

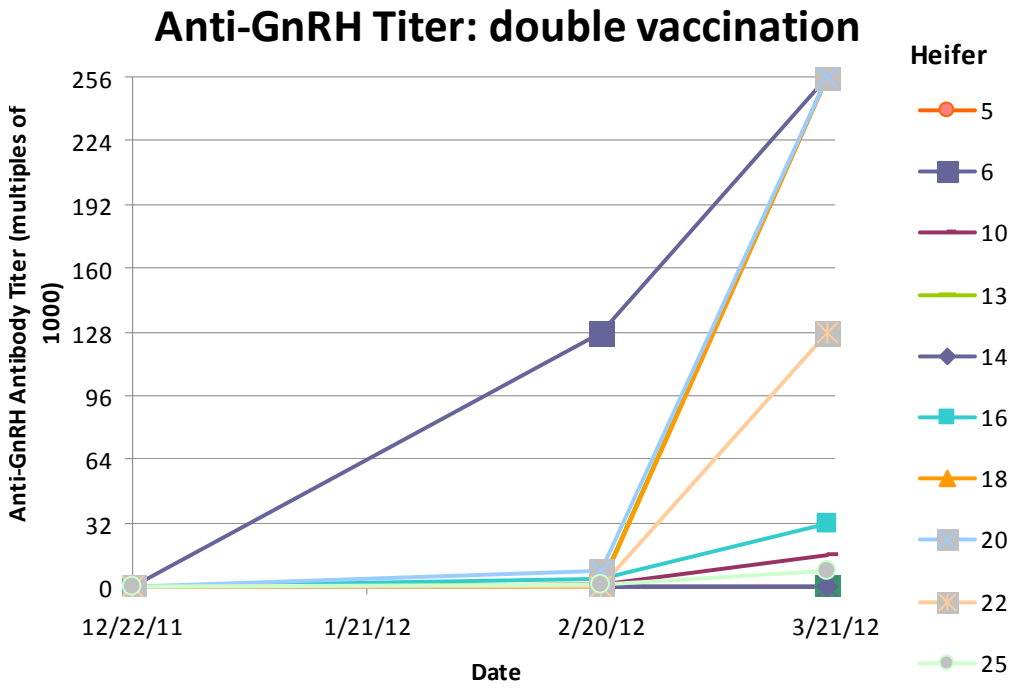

Figure 6. Longitudinal changes in anti-GnRH antibody titres for individual heifers that received a double GonaCon™ vaccination on Day 0 (12/22/11) and Day 60 (2/20/12).

Ovarian follicular status

Results for ovarian follicular status are shown in Tables 10 to 12. Control heifers showed cyclic ovarian activity throughout the project (Table 10).

For heifers that received a single GonaCon™ vaccination, only one heifer (Heifer 4, Table 11) showed suppressed ovarian activity. This heifer did not have anti-GnRH antibody titres that were noticeably different to titres for other heifers that received a single GonaCon™ vaccination (Table 8).

Heifers that received a double GonaCon™ vaccination did not have suppressed ovarian activity at 60 days after primary vaccination, except for Heifer 6 (Table 12). It is noteworthy that Heifer 6 had significant anti-GnRH antibodies at Day 60 (Table 9). After secondary vaccination on Day 60, 9/9 heifers had suppressed ovarian activity on Day 89 and 5/9 heifers continued to have suppressed ovaries to Day 391 (331 days after secondary vaccination) (Table 12). For the latter heifers, 4/5 had high anti-GnRH antibody titres after secondary vaccination and 1/5 heifers had a modest titre. Two heifers with high anti-GnRH titres at Day 89 returned to active ovarian activity after Day 126 and before Day 154.

Table 10. Ovarian follicular scores for individual control heifers.

Heifer	Ovarian score							
	Day of study							
	0 Dec 2011	60 Feb 2012	89 Mar 2012	126 Apr 2012	154 May 2012	209 Jul 2012	316 Nov 2012	391 Jan 2013
11	3	3	3	3	3	3	3	3
12	3	3	3	3	3	3	3	3
17	3	3	3	3	3	3	3	3
21	3	3	3	1	3	3	3	3
26	3	3	3	3	3	3	3	3

Table 11. Ovarian follicular scores for individual heifers that received a single GonaCon™ vaccination on Day 0.

Heifer	Ovarian score							
	Day of study							
	0 Dec 2011	60 Feb 2012	89 Mar 2012	126 Apr 2012	154 May 2012	209 Jul 2012	316 Nov 2012	391 Jan 2013
1	3	3	3	3	3	2		
3	3	3	3	3	3	3		
4	3	2	3	1	1	1	1	1
7	3	3	3	1	3	3		
8	3	3	3	3	2	2		
9	3	3	3	3	3	3		
19	3	3	3	3	3	3		
23	3	3	3	3	1	3		
24	2	3	3	3	2	3		
27	3	1	2	3	3	3		

Table 12. Ovarian follicular scores for individual heifers that received a double GonaCon™ vaccination on Day 0 and Day 60.

Heifer	Ovarian score							
	Day of study							
	0 Dec 2011	60 Feb 2012	89 Mar 2012	126 Apr 2012	154 May 2012	209 Jul 2012	316 Nov 2012	391 Jan 2013
5	3	3	1	1	1	1	1	1
6	3	1	2	1	1	1	2	1
10	3	3	2	3	3	3		
13	3	3	1	2	3	3		
14 †	3	3	3	3	3	3		
16	3	3	1	1	1	2	1	1
18	3	3	1	2	3	3		
20	3	3	1	1	1	1	1	1
22	3	3	1	1	1	1	1	1
25	3	2	1	2/3*	3	3		

† heifer did not receive a primary vaccination

5. Discussion

This project examined the immune and reproductive responses of heifers to single and double vaccination with the anti-fertility vaccine GonaCon™. This vaccine had been shown to suppress fertility long-term after a single vaccination in a range of species including wildlife, domestic animals and production animals (22-27, 29). GonaCon™ incorporates *Mycobacterium avium* (*M. avium*) which is a key component since *M. avium*, and closely related organisms, are endemic in many regions. Hence, single vaccination with GonaCon™ evokes immune memory to Mycobacterium-type organisms and the ensuing immune response includes the generation of anti-GnRH antibodies. The anti-GnRH antibody response is significant and sustained and is associated with an immunocontraceptive response that can be maintained for 3 to 5 years (22-27, 29). In the first phase of the current project, single vaccination with GonaCon™ was investigated in heifers in southern Queensland. Heifers did not show anti-GnRH antibodies after single vaccination and they were retrospectively found not to have anti-*M. avium* titres at the time of vaccination. It was concluded that the heifers had not been pre-exposed to *M. avium* and/or closely related organisms and therefore had no immune memory when vaccinated with GonaCon™, which relies on prior exposure to these organisms. *M. avium paratuberculosis* is the causative agent for Johne's Disease which occurs in southern Australia but is very uncommon in northern Australia, which very likely explains the lack of a response to single vaccination with GonaCon™.

Single vaccination with GonaCon™ also failed to induce effective anti-GnRH antibody titres in the present project.

In contrast, double vaccination with GonaCon™ evoked an anti-GnRH antibody response after secondary vaccination, with particularly high titres in 6/9 heifers. This was associated with suppressed ovarian activity after secondary vaccination. Ovarian follicular growth remained suppressed for > 300 days in 5/9 heifers. Of the latter heifers, 4/5 had high anti-GnRH antibody titres after secondary vaccination and 1/5 heifers had a relatively modest titre. However 2/9 heifers with high anti-GnRH titres returned to normal ovarian activity by Day 156.

The measurement of anti-GnRH antibody titres to Day 300 was beyond the scope of the project. It is possible that continued suppression of ovarian activity in a proportion of heifers vaccinated twice with GonaCon™ was due to the maintenance of anti-GnRH antibody titres in blood above a threshold required to bind and neutralise the majority of GnRH released from the brain. Another possibility was that vaccination with GonaCon™ caused a permanent morphological disruption of GnRH neuron terminals which originate in the hypothalamus and terminate in the median eminence. Boars immunised against GnRH showed tissue lesions in the median eminence and there was a relationship between the presence of lesions and the magnitude of the immune response (28). It was proposed that anti-GnRH antibodies (IgG antibodies) were involved in lesion formation although potential mechanisms were not explored (28). It was also presumed that the lesions disrupted the release of GnRH from GnRH neuron terminals and/or the movement of GnRH into the portal capillary system which bathes the median eminence and transports GnRH to the anterior pituitary gland.

A long-term suppression of testicular function was observed in a proportion of bull calves immunised against GnRH (6) and it is possible that lesions similar to those described in boars (28) may have also occurred in bull calves. Likewise, the heifers that showed sustained suppression of ovarian activity after double vaccination with GonaCon™ may have undergone a disruption to the morphological integrity of the GnRH neuronal system. For the heifers that showed continued suppression of ovarian activity, 4/5 had high anti-GnRH antibody titres and 1/5 had a more moderate titre after secondary vaccination, and it is likely that a range of outcomes (direct antibody titre, morphological lesion, other mechanisms) explain longer-term suppression of reproductive function after active immunisation against GnRH. High anti-GnRH antibody titres, either transient or sustained, would appear to be a general pre-requisite for continued suppression of gonal function. However, this may not be obligatory as 1/10 heifers that received a single GonaCon™ vaccination, and which had a low anti-GnRH antibody titre, showed long-term suppression of ovarian activity. This would suggest that there are individual differences in the interrelationships between anti-GnRH antibody titres and impact on reproduction. Notwithstanding, the goal in GnRH immunisation should be to achieve a maximal immune response and high anti-GnRH antibody titres in blood.

Heifers given a single vaccination received 3 mg GonaCon™ and heifers given a double vaccination received 2 mg GonaCon™ for primary injection and 1 mg GonaCon™ for secondary injection. It had been anticipated that all heifers given a double vaccination would show a significant immune response after secondary injection and would have suppressed ovarian activity. This expectation was based on reports of sustained suppression of fertility for a range of species after immunisation with GonaCon™ (7, 23-27, 29). Contrary to expectation, 3/9 heifers showed a relatively low anti-GnRH antibody response after secondary vaccination. It is possible that the use of 1 mg GonaCon™ for secondary immunisation was at the margin for the amount of GonaCon™ required to elicit an immune response in all heifers vaccinated. Bison heifers that received a single immunisation with 1.8 mg GonaCon™ had relatively high anti-GnRH antibody titres longer-term and did not conceive for 12 months after immunisation (25). The study in bison is perhaps most comparable to cattle and the evaluation of higher doses of GonaCon™ in a 2-vaccination schedule would seem to be worthwhile in heifers.

A GnRH vaccine that induced a sustained immunological and anti-fertility response in cattle at a reasonable net cost would be ideal for application is northern Australia. In the absence of such a vaccine, a 2-vaccination schedule with GonaCon™ would still have application with current cattle management if applied at branding and weaning. This would only be acceptable if the response to GonaCon™ was maintained for at least 12 months and ideally 2 to 3 years. One application in heifers would be for young heifers not destined for potential breeding but rather specific preparation for turn-off into different production systems. There would be broad application to replace castration in bulls.

6. Conclusion

This project sought to determine whether double vaccination of heifers with the immunocontraceptive vaccine GonaCon™ would consistently induce significant anti-GnRH antibody titres that would be maintained long-term, and be associated with suppressed ovarian follicular activity.
It was found that 6/9 heifers had high anti-GnRH antibody titres after secondary vaccination and 5/9 heifers continued to have suppressed ovarian activity 330 days after secondary vaccination when the project ended. The conclusion from the project was that higher doses of GonaCon™ should be evaluated in heifers for primary and secondary vaccination (potentially 5 mg and 3 mg, respectively). The neutralisation of GnRH should be pursued as an alternative to spaying and castration as it is not a gender-specific approach and the same technology would have application in both female and male cattle.

Appendix 1

GonaCon™ vaccine formulation

The GonaCon™ immunocontraceptive vaccine consists of mammalian gonadotrophin releasing hormone (GnRH) conjugated to keyhole limpet hemocyanin (KLH) and emulsified in AdjuVac™ adjuvant. The GnRH/KLH conjugate and AdjuVac™ adjuvant are prepared in a water and mineral oil emulsion.

Each 1.0 ml dose of GonaCon™ contains the following:

GnRH/KLH Conjugate (1,000 ug)	GnRH	300 µg
	KHL	660 µg
	Mollusk Stabilizing Buffer (MSB)	
	(Phosphate buffered saline)	21.30 mg
	Sodium chloride	0.54 mg
	Potassium chloride	3.06 mg
	Sodium phosphate, dibasic	0.53 mg
	Potassium phosphate, monobasic	5.33 mg
	Sucrose	0.50 ml
	Sterile, ultrapure water	
AdjuVac™ adjuvant	*Mycobacterium avium*	170 µg
	Light mineral oil	0.45 ml
	Mannide monooleate	0.05 ml

Appendix 2

Ovarian folliculogenesis and ovarian status - overview

The essential features of ovarian folliculogensesis and ovarian status in the cow can be summarised as follows:

Ovarian folliculogenesis

1. Ovarian follicular growth commences with the transition of a follicle from the resting pool of primordial follicles to a primary follicle;
2. The progression of follicles from the primary stage to early antral stage requires around 3 to 4 months in the cow and is considered to be independent of gonadotrophin support;
3. At the gonadotrophin-dependent stage, follicles require stimulation by follicle stimulating hormone (FSH) to enter the final stages of follicular growth and development, and potential progression to ovulation;
4. It is typical for a cohort of early antral follicles to respond to FSH at the same time and commence the final stages of development as a follicular wave (usually 7 to 10 follicles);
5. During the final stages of follicular development, follicles switch dependency from FSH to luteinising hormone (LH); and
6. The dominant follicle in a follicular wave progresses to a pre-ovulatory follicle which requires exposure to the pre-ovulatory surge release of LH in order to ovulate and develop into a corpus luteum.

Ovarian status

1. *Suppressed follicular growth at early antral stage*
   Follicles grow from the primordial to early antral stage and do not proceed further; in Zebu cows early antral follicles are typically ≤ 5 mm in diameter; the failure of follicles to progress beyond the early antral stage is due to the lack of stimulation by FSH (i.e. the absence of a transient rise of FSH in circulation);

2. *Follicular growth without ovulation*
   Follicles progress from the early antral stage to late antral stages but do not ovulate; this ovarian state can be interpreted to be indicative of the transition of follicles beyond the FSH dependent stage but there is a lack of LH to promote continued follicular development, and there is also absence of the pre-ovulatory surge release of LH to induce ovulation; and

3. *Follicular growth with ovulation*
   Follicles progress through all stages of development and the dominant ovarian follicle ovulates and develops into a corpus luteum; this ovarian state is interpreted to indicate that reproductive endocrine function, including the patterns and sequencing of FSH and LH secretion, is normal.

Ovarian status scores

Ovarian status can be scored as follows:

Ovarian status	**Typical follicle size in Zebu (*Bos indicus*)**	**Score**
Suppressed follicular growth at early antral stage	≤ 5 mm	1
Follicular growth without ovulation	6 to > 10 mm *	2
Follicular growth with ovulation and corpus luteum **	> 10 mm	3

* follicles can grow to a relatively large size and not ovulate
** cows that show ovulation and have a corpus luteum are considered cyclic or oestrus

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