

1 **Early post parturient changes in milk acute phase proteins**

2 Funmilola C Thomas<sup>1</sup>, Mary Waterston<sup>2</sup>, Peter Hastie<sup>3</sup>, Hayley Haining<sup>3</sup> and P David  
3 Eckersall<sup>4\*</sup>

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5 <sup>1</sup> Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine,  
6 Federal University of Agriculture, Abeokuta, Nigeria

7 <sup>2</sup>Institute of Infection, Immunity and Inflammation, College of Veterinary, Medical and Life  
8 Sciences, University of Glasgow, Glasgow, United Kingdom

9 <sup>3</sup>School of Veterinary Medicine, University of Glasgow, Glasgow United Kingdom

10 <sup>4</sup> Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow,  
11 Glasgow, United Kingdom.

12 **Short title: Acute phase proteins in post parturient milk**

13 Email addresses:

14

15 **\*Correspondence: P. David Eckersall**

16 Institute of Biodiversity, Animal Health and Comparative Medicine, School of Veterinary  
17 Medicine, 464 Bearsden Road, Glasgow, United Kingdom. G61 1QH

18 *E-mail:* [David.Eckersall@glasgow.ac.uk](mailto:David.Eckersall@glasgow.ac.uk)

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## 21 **Summary**

22 The periparturient period is one of the most critical periods in the productive life of a dairy  
23 cow, and is the period when dairy cows are most susceptible to developing new  
24 intramammary infections (IMI) leading to mastitis. Acute phase proteins (APP) such as  
25 haptoglobin (Hp), mammary associated serum amyloid A3 (M-SAA3) and C-reactive protein  
26 (CRP) have been detected in milk during mastitis but their presence in colostrum and milk in  
27 the immediate postpartum period has had limited investigation. The hypothesis was tested  
28 that APP are a constituent of colostrum and milk during this period. Enzyme linked  
29 immunosorbent assays (ELISAs) were used to determine each APP's concentration in  
30 colostrum and milk collected daily from the first to tenth day following calving in 22  
31 Holstein-Friesian dairy cows. Haptoglobin was assessed in individual quarters and composite  
32 milk samples while M-SAA3 and CRP concentration were determined in composite milk  
33 samples. Change in Hp in relation to the high abundance proteins during the transition from  
34 colostrum to milk were evaluated by 1 and 2 dimension electrophoresis and western blot. In  
35 80% of the cows all APPs were detected in colostrum on the first day following parturition at  
36 moderately high levels but gradually decreased to minimal values in the milk by the 6<sup>th</sup> day  
37 after calving. The remaining cows (20%) showed different patterns in the daily milk APP  
38 concentrations and when an elevated level is detected could reflect the presence of IMI.  
39 Demonstration that APP are present in colostrum and milk following parturition but fall to  
40 low levels within 4 days means that elevated APP after this time could be biomarkers of post  
41 parturient mastitis allowing early intervention to reduce disease on dairy farms.

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43 **Keywords:** post-calving, haptoglobin, mammary associated serum amyloid A3, C-reactive  
44 protein, dairy cow, mastitis

45 The periparturient period is one of the most critical periods in the productive life of a dairy  
46 cow characterized by an increased susceptibility to diseases (Trevisi *et al.* 2010). This has  
47 been attributed to negative energy balance (NEB) and the associated immune suppression at  
48 the puerperal period (Waldron & Revelo, 2008; Hiss *et al.* 2009). During this periparturient  
49 period, immune suppression increased serum levels of metabolic and endocrine markers such  
50 as prostaglandins (Yuan *et al.* 2013), cortisol, ketone bodies (for example  $\pm$ -butyric acid) and  
51 non-esterified fatty acids (NEFA) occur. Hypoglycaemia and hypocalcaemia have also been  
52 defined as markers of metabolic stress during this period in dairy cows (Waldron & Revelo,  
53 2008; Esposito *et al.* 2014). Further studies have also shown inflammatory markers such as  
54 the acute phase proteins, haptoglobin (Hp) and serum amyloid A (SAA) to be increased in  
55 maternal serum during the periparturient period (Trevisi *et al.* 2012) while these acute phase  
56 proteins are known to be elevated in serum and milk during mastitis (Eckersall *et al.* 2006).

57 Earlier, Morimatsu *et al.* (1991) demonstrated an increase in bovine serum C-reactive protein  
58 (CRP) associated with onset of lactation in Holstein cows. Furthermore Schrodl *et al.* (1995)  
59 identified CRP in bovine colostrum and milk, and suggested this could be passively  
60 transferred to the serum of colostrum fed calves (Schroedl *et al.* 2003). In humans CRP is a  
61 major APP and has been shown to be increased in serum at the post-partum period which has  
62 been attributed to the trauma associated with childbirth, with concentration dropping back to  
63 baseline values by the 5<sup>th</sup> day post-partum (Fetherston *et al.* 2006).

64 It is well established that colostrum and milk in the immediate post-partum period contains a  
65 large repertoire of immunological proteins such as immunoglobulin G (IgG). In addition, the  
66 APP, M-SAA3 (McDonald *et al.* 2001) and alpha-1 glycoprotein (AGP) (Ceciliani *et al.*  
67 2005), have been observed to be elevated in colostrum and this has been suggested to be due  
68 to physiological roles of these proteins in providing immunity to infectious diseases for the  
69 new-born. Hiss *et al.* (2009) also showed that Hp is high in milk of metabolically stressed

70 transition dairy cows when the concentration of Hp in weekly milk samples was determined  
71 in the periparturient milk for up to 12 weeks post-partum.

72 Mastitis is one of the prevalent conditions in dairy cows, which arises most frequently during  
73 the periparturient period, compared to at any other period of the life of a dairy cow (Waldron  
74 & Revelo, 2008). The use of APP in milk is gaining prominence as markers for mastitis  
75 diagnosis in dairy cows; therefore it would be of value to evaluate their concentrations in the  
76 periparturient period so that any change due to an intra mammary infection (IMI) can be  
77 identified. Due to the fact that the immediate post-parturient milk (colostrum) contains high  
78 concentrations of immune related proteins, there is a challenge in readily diagnosing new  
79 infections in the mammary gland with regards to differentiating the physiological from the  
80 pathological increases of marker proteins but it is not clear if the APP are present in this  
81 milieu. Understanding the physiological change, if any, in the milk APP concentrations  
82 during the periparturient period so that pathophysiological conditions can be identified would  
83 allow prompt identification of new mastitis cases developing in postpartum udders/quarters in  
84 order to readily initiate treatment. Accordingly, the hypothesis was tested that APP such as  
85 Hp, M-SAA3 and CRP are present in colostrum and milk following parturition, potentially  
86 affecting the diagnosis of mastitis in dairy cows during this period. The hypothesis was tested  
87 by determining the concentrations of the APP in milk samples collected from cows calving  
88 on a commercial dairy farm. Changes in the high abundance protein of milk during this  
89 period were observed for comparison by electrophoresis.

## 90 **Materials and Methods**

### 91 *Sample collection*

92 Daily quarter milk samples were collected from Holstein-Friesian cows, starting from the  
93 first milking immediately following parturition and then daily during morning milking for up  
94 to ten days post-partum. Cows were from a commercial dairy farm in the west of Scotland  
95 (Cochno Research Farm, University of Glasgow) and comprised all cows calving within a  
96 period of six months (January to June, 2013). The cows were fed a total mixed ration (TMR)  
97 and had lactation number (parity) from 1 to 4. Approximately 15 ml milk was collected from  
98 each udder quarter after discarding the first strips of milk following teat disinfection. The  
99 sample collection procedure has been described in Thomas *et al.* (2015). Samples were stored  
100 at -20°C until analysed with a maximum of 4 weeks storage before Hp assay but with up to 6  
101 months for M-SAA3 and CRP assays due to assay kit availability.

102 Twenty four (24) cows calved within the 6 months study period on the farm; however, 2  
103 cows were excluded from analyses due to systemic condition requiring veterinary treatments.  
104 Haptoglobin was analysed in milk samples collected from individual quarters (n=84, four  
105 cows lacked one functional quarter each) of each of the 22 calving cows, daily for the 10 days  
106 duration of sampling. Out of 22 cows whose milk samples were analysed, 1 cow had missing  
107 samples for day 10, while another for days 2 and 7-10. Daily composite milk samples were  
108 derived by mixing equal volumes from the daily quarter samples collected for each cow and  
109 were assayed for Hp, M-SAA3 and CRP. Samples were stored at -20°C and for assay were  
110 thawed at room temperature, thoroughly mixed by vortexing and diluted in the respective  
111 assay/wash buffer for Hp, M-SAA3 and CRP (n=22/day for 10 days). Haptoglobin was  
112 assayed in all individual quarter milk samples (n=575) due to the availability of a relatively  
113 affordable in-house ELISA, compared to commercial kits that had to be purchased for assay  
114 of M-SAA3 and CRP which were therefore only assayed in composite samples. The study

115 was approved by the ethics committee of the University of Glasgow, School of Veterinary  
116 Medicine.

#### 117 *Acute phase protein assays*

118 Haptoglobin; Purified rabbit anti-bovine haptoglobin IgG (*Life Diagnostic Inc., West Chester,*  
119 *USA*) was conjugated to alkaline phosphatase (ALP) (*Innova biosciences*) according to the  
120 manufacturer's instructions. Sandwich ELISA procedure was carried out as described by  
121 Thomas et al. (2015).

122 Mammary associated serum amyloid A3: Tridelta Development Ltd supplied the Phase™  
123 Range SAA ELISA kit (sandwich ELISA kit for measuring multispecies SAA, Phase™  
124 Range by Tridelta Development Ltd (Kildare, Ireland) and performed as described in Thomas  
125 *et al.* (2015).

126 C-reactive protein: Cow C-reactive protein (CRP) ELISA kits for assay of milk CRP were  
127 supplied by the *Life Diagnostics Inc.* (West Chester, USA). The assay was based on a solid  
128 phase sandwich ELISA format, and comprised of primary anti-bovine CRP antibodies  
129 immobilized to the wells of a 96-well microtitre plate and secondary antibodies against the  
130 anti-bovine CRP conjugated to horse radish peroxidase (HRP) and performed as described in  
131 Thomas *et al.* (2015).

#### 132 *1 & 2 Dimensional Gel Electrophoresis*

133 Daily composite milk samples (day 1 to 10) of calving cows were run on 1DE SDS PAGE as  
134 described by Braceland et al. (2014) to identify changes in the high abundance proteins of  
135 milk. Day 1 sample (colostrum) and day 10 milk were each resolved on 2DE SDS PAGE as  
136 described by Braceland et al. (2013) to further depict the transition from colostrum to milk.

137 *Western blot*

138 Quarter samples from a cow that showed irregular fluctuation in APP content in the days post  
139 calving, were examined by Hp western blotting following 1DE.

140 Briefly, 1DE was carried out as described above after which the proteins on the gels were  
141 blotted onto a nitrocellulose membrane (NCM) as described in Braceland *et al.* (2014) using  
142 IgG fraction of rabbit anti-bovine Hp conjugated to alkaline phosphatase (Thomas *et al.* 2015)  
143 for incubation and Pierce™ NBT/BCIP (Thermo Scientific, UK) to develop the colour of Hp  
144 bands (Braceland *et al.* 2014).

145 *Statistical analyses*

146 Tests for normality were carried out on all APP data using the Kolmogorov–Smirnov and  
147 Shapiro–Wilk tests along with normal probability plots and quantile-quantile (Q-Q) plots and  
148 Spearman’s rho test was used to assess the correlation between the 3 APP using the statistical  
149 package for social sciences (SPSS) software version 21 (IBM SPSS, Portsmouth, UK). The  
150 daily milk sample APP concentrations were found to be not normally distributed and A non-  
151 parametric test (Mann Whitney) was employed to determine the days after parturition that the  
152 composite milk APP became significantly different from values in milk collected on day 10  
153 using Minitab 17, Minitab Ltd., Coventry UK. A P-value of <0.05 was considered  
154 significant.

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## 156 **Results and Discussion**

157 The investigation of the APP in colostrum as it changed to milk has found APP are present in  
158 colostrum but within a few days their concentration is reduced to minimal levels. To put this  
159 in context of the concurrent changes in high abundance milk proteins, Figure 1 shows the  
160 SDS-PAGE gel of daily milk samples with the proteins identified by comparison to published  
161 gel images and Mw reports (Jovanovic *et al* 2007; O'Mahony 2014; Edwards & Jameson,  
162 2014). The progressive reduction of IgG and albumin in composite samples from an during  
163 the first 10 days post parturition and the increase in  $\alpha$ -lactalbumin,  $\beta$  lactoglobulin and  
164 lactoferrin was apparent. This was also demonstrated by the comparison of composite milk  
165 samples from day 1 and day 10 post parturition on 2 DE (Figure 2A and 2B) which also are  
166 in agreement with the known proteomes of colostrum and milk (Hogarth *et al.* 2004;  
167 Hernández-Castellano 2014). These electrophoretic separations demonstrate the change in  
168 high abundance protein of colostrum as it converts to milk (Hernández-Castellano *et al.*  
169 2014) and also show the inability of this approach to identify low abundance proteins  
170 including the APP so that measuring changes in their concentration in colostrum or milk  
171 needs more sensitive methodology such as the immunoassays used in this investigation.

172 Therefore it was by using an ELISA that the concentration of Hp in individual quarter milk  
173 samples could be quantified revealing a pattern of progressively decreasing median values of  
174 Hp concentration in milk with days post-calving (Figure 3).. The median concentration of Hp  
175 in individual colostrum/milk after calving fell from 13.5  $\mu\text{g/ml}$  on day 1 to 4.9  $\mu\text{g/ml}$  on day  
176 4 and thereafter remained at 3-4  $\mu\text{g/ml}$  until day 10. When examining the individual quarter  
177 milk samples this pattern was followed in 63 out of the 84 quarters (75 %) examined. In 8 of  
178 the quarter milk samples the Hp was  $>200 \mu\text{g/ml}$  on day 1 to 4 which would be equivalent to  
179 levels found in milk from quarters with IMI caused by *Escherichia coli* or *Arcanobacterium*



180 *pyogenes* where median levels of 244 µg/ml and 440 µg/ml respectively have been found  
181 (Pyorala et al 2011). Among the quarter milk samples that did not show this general pattern,  
182 4 quarters had undetectable Hp in all samples (day 1 to 10 post calving) while two other  
183 patterns of variations were observed in the remaining 17 quarters. There were 4 quarter milk  
184 samples in which Hp increased above the levels found on days 1-3 post-calving instead of  
185 dropping in concentration. These quarters are possibly developing IMI or undergoing other  
186 forms of inflammatory stimulus that can influence the occurrence of an APR but in this  
187 retrospective study it was not possible to confirm the presence of IMI. However, monthly  
188 SCC assessments indicated that all of the cows sampled were mastitis free. There were also  
189 quarters (n=13) with irregular fluctuations in Hp concentration but with a general downward  
190 trend with 4 of these shown in Figure 4. The Hp western blot (Figure 5) of an individual  
191 quarter's samples show the lower Mw  $\alpha$  subunit and the higher Mw  $\beta$  subunit of Hp  
192 decreasing, though with some fluctuation during the day 1 – 10 post calving period,  
193 confirming the immunoassay results.

194 In the composite milk samples, Hp was moderately high (median 19.6 µg/ml, n=22) in the  
195 first days post-calving milk (colostrum), and gradually dropped within 3 to 5 days after  
196 parturition to a median of 5.2 µg/ml on day 5, (n=22) (Figure 6). By day 4 the median  
197 concentration of Hp had dropped and was not significantly different from that of day 10  
198 samples. These were equivalent to the range of Hp in composite milk samples found on a  
199 commercial dairy farm (Thomas *et al.* 2015) which had a median of 3.46 µg/ml and a range  
200 of 0.4 – 55.46 µg/ml. Composite milk Hp has been found to be raised to 101 µg/ml in cows  
201 with chronic sub-clinical mastitis (Gronlund et al 2005) so that up to day 3 after calving the  
202 concentration of Hp is equivalent of cows with such a level of infection. Experience of  
203 monitoring Hp in milk of cows in an experimental *S. aureus* mastitis model has shown that  
204 concentrations in the region of > 150 µg/ml are found (Eckersall *et al.* 2006). Thus in the

205 first days post-calving the expression and secretion of Hp can in some cows be in the same  
206 order as during mastitis.

207 Individual milk samples were not assayed for M-SAA3 but similar to Hp in composite milk ,  
208 an elevated median M-SAA3 concentration in composite milk was observed for day 1 and 2  
209 (medians of 17 and 5  $\mu\text{g/ml}$  respectively) which was significantly higher than values for day  
210 10 milk and fell as the days progressed reaching by day 4, levels which was not significantly  
211 different from day 10 milk M-SAA3 (Figure 7). The medians for day 1 and 2 are in the same  
212 order as composite milk from cows with sub clinical mastitis where concentrations up to 25  
213  $\mu\text{g/ml}$  were described (Gronlund et al 2005). By day 10 the MSAA3 concentrations were  
214 equivalent to composite milk samples found on a commercial dairy farm (Thomas *et al.*  
215 2015) which had a median of 1.17  $\mu\text{g/ml}$  and a range of 0.6 – 50.13  $\mu\text{g/ml}$ . The moderately  
216 raised levels of M-SAA3 observed in composite milk on day 1-3 , are also consistent with the  
217 reports by McDonald *et al.* (2001) where elevated levels of M-SAA3 in bovine colostrum  
218 significantly dropped by day 4 post-calving. Similar to the observations for Hp, the  
219 concentration of M-SAA3 in colostrum is above the basal level in healthy milk and is  
220 comparable, allowing for the dilution effect of composite milk to quarters with mastitis, in  
221 which the concentration of M-SAA3 can be  $>100 \mu\text{g/ml}$  (Eckersall *et al.* 2006). The  
222 concentration of M-SAA3 is known to reduce on storage at  $-20^{\circ}\text{C}$  by around 20% within 7  
223 days (Tothova *et al.*, 2012) but thereafter to stabilise so that the concentrations here, measured  
224 after around 6 months of storage may be affected. However all samples were stored similarly  
225 and the relative change in M-SAA3 post parturition can be accepted.

226 Milk CRP was found to have similar aspects to Hp and M-SAA3 in post-calving composite  
227 milk, by being raised on the first 1 to 3 days and then gradually falling to the concentration  
228 found in healthy cows (Figure 8) (Thomas *et al.* 2015). By day 3, the milk CRP concentration  
229 was not significantly different from day 10 with concentrations falling within the range for

230 healthy milk samples as observed in Thomas *et al.* (2015) which had a median of 24.56  
231 ng/ml. and a range of 1.8 – 172 ng/ml. The findings on milk CRP confirms the reports of  
232 Schroedl *et al.* (2003) of the presence of CRP in bovine colostrum. According to Lee *et al.*  
233 (2003) bovine serum CRP levels correlated with lactation status, being highest during peak  
234 lactation period (2-4 months of pregnancy) while in the study of Zimmermann *et al.* (1998)  
235 plasma CRP in cows were increased post-partum. However, there have been no previous  
236 reports of the daily variation of CRP in bovine milk from the day of parturition to 10 days  
237 after.

238 Previous studies have reported increases in APP in serum during the first week(s) post  
239 calving (Uchida *et al.* 1993; Alsemgeest *et al.* 1995; Humblet *et al.* 2006), but few studies  
240 have investigated the effect of parturition on different milk APP (McDonald *et al.* 2001;  
241 Ceciliani *et al.* 2005). The concentration of serum Hp in this period has been used to show  
242 the presence of stress the cow is undergoing as part of the parturition process. Variations in  
243 milk Hp, M-SAA3 and CRP at this early post-partum period could help to assess for presence  
244 of new post-calving IMI. The elevated level of the APP in milk in the first few days post  
245 calving suggests a role for them in colostrum by conferring maternal protection to the new  
246 born. On the other hand, it may be due to the stress induced by parturition and its effects  
247 extending to the mammary gland. It was found that the major pattern observed for these APP  
248 during the post-partum period, followed a similar trend to that observed for milk somatic cell  
249 counts (SCC) in the studies of Barkema (1999) and Sargeant *et al.* (2001) in the early  
250 lactation period. This observed pattern of SCC could explain the Hp pattern, as studies have  
251 shown that somatic cells such as neutrophils are a major source of the Hp found in milk (Lai  
252 *et al.* 2009).

253 The source of the APP seen in the post parturient milk was not identified in this study, but  
254 there have been reports of local synthesis of Hp (Hiss *et al.* 2004) and M-SAA3 (McDonald

255 *et al.* 2001) in the mammary gland tissue. There are no reports on the source of CRP in  
256 bovine milk, and it is possible that the CRP in colostrum and milk arise either from passive  
257 transfer from the circulation, or from local production in the mammary gland.

258 The milk concentration of Hp, M-SAA3 and CRP when compared in composite milk samples  
259 from the 22 cows showed significant correlations between samples (Table 1). Although there  
260 was a general similarity in the distribution of the 3 APP, small differences were also  
261 observed, for example, CRP fell back to basal levels more rapidly (day 4) than M-SAA3 (day  
262 5) and Hp (day 6). Median concentration in CRP showed a late (day 9) increase in 2 samples  
263 which affected the median/range results. On a practical issue for application of the APP as  
264 biomarkers of post-parturition stress in the mammary gland, the Hp ELISA being in-house-  
265 developed assay was more economical for analysis of all individual quarter samples, whereas  
266 the M-SAA3 and CRP assays were limited to the composite samples. Extending the  
267 investigation of individual quarter samples to all of the APP would be valuable especially to  
268 assess their sensitivity and specificity for IMI and if multiplex immunoassay could be  
269 developed to allow detection of all APP in one sample. The use of composite milk dilutes the  
270 concentration of APP as seen here with the maximum Hp being over 800 µg/ml in quarter  
271 milk and 350 µg/ml in composite milk. It is likely that the concentrations of M-SAA3 and  
272 CRP in individual quarter samples would have been higher than the levels found in composite  
273 milk.

274 Other milk APP have been identified as being high in colostrum and decrease in milk post  
275 calving such as alpha-1 acid glycoprotein (Ceciliani *et al.* 2005), lactoferrin and transferrin  
276 (Sanchez *et al.* 1988). However to the best of our knowledge this is the first report of daily  
277 variation in the levels of Hp, M-SAA 3 and CRP in colostrum and milk over the first 10 days  
278 immediately after parturition. A recent report has demonstrated the variation in many low  
279 abundance proteins of post-parturient milk using a proteomic approach (Zhang *et al.* 2015)

280 and demonstrated changes in haptoglobin and SAA1 and SAA3 (equivalent to M-SAA3  
281 reported here) but CRP was not detected.

282 The finding that Hp, M-SAA3 and CRP are raised in colostrum and milk during the first few  
283 days post calving, would mean that caution should be used in the interpretation of results in  
284 using them for detecting IMI during this period in a dairy cow's cycle. However, APP assay  
285 will be valuable for detecting new IMI in the periparturient period, after the first few days (4<sup>th</sup>  
286 day) after calving when a drop in APP would be expected in the absence of IMI. It would be  
287 interesting to compare the APP profile in cows/udders developing new IMI during the  
288 immediate parturition period with the profile observed in 80% of cows from this study which  
289 were mastitis free. This would probably better highlight differences that could enhance the  
290 value of use of APP in recognising new IMI post-partum, and should be a subject for future  
291 research. There was individual variation in the concentration of the APP in the individual  
292 and composite samples with, for a number of samples, the concentration of APP in colostrum  
293 being as high as in mastitis. Whether there is any advantage in having a high level of APP in  
294 the colostrum to the calf or to the cow would be worthy of further investigation. Adoption of  
295 the APP assays to a rapid measurement format will be required before they can be generally  
296 used. Indeed a rapid test for any of the APP may be a better cow-side test for IMI than CMT  
297 in detecting the host response to major-pathogen mastitis in the immediate postpartum period  
298 (Dingwell *et al.* 2003).

299 In conclusion, moderately elevated concentrations of Hp, M-SAA3 and CRP have been found  
300 in colostrum and milk in the post parturient period. The concentrations of the APP fall to  
301 basal levels by the 4<sup>th</sup> day post calving. High or increasing levels of these biomarkers beyond  
302 the 4<sup>th</sup> day post calving could be suggestive of an on-going or a new IMI and could enhance  
303 current diagnostic procedures for this condition.

304

305

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419 **Table 1:** Correlation between Hp, MSAA3 and CRP in composite milk from cows (n=22)  
 420 over 10 time points (day 1-10)

421

		MSAA3	CRP	Hp	
Spearman's rho	MSAA3	Correlation	1.000	.431**	.630**
		Coefficient			
		Sig. (2-tailed)		.000	.000
	CRP	Correlation		1.000	.522**
		Coefficient			
		Sig. (2-tailed)			.000
	Hp	Correlation			1.000
		Coefficient			
		Sig. (2-tailed)			

422 \*\* Correlation is significant at the 0.01 level (2-tailed).

423

424 **Legends to figures**

425 **Figure 1:** 1DE reducing gel electrophoretogram of immediate post-partum milk samples (day  
426 1-10) pooled from healthy udder of cow A. Ig (immunoglobulin), Bovine Lf (bovine  
427 lactoferrin),  $\pm$ S<sub>2</sub>-CN (alpha S<sub>2</sub> casein), <sup>2</sup>-CN (beta casein), <sup>o</sup>-CN (kappa casein), <sup>2</sup>-LG (beta  
428 lactoglobulin),  $\pm$ -LA (alpha lactalbumin), DPC (days post-calving), kDa (kilo Dalton).

429 **Figure 2A:** 2DE reducing gel of pooled (quarters) colostrum (day 1 post-calving)  
430 sample. Isoelectric range pH 3-10, from one representative calving cow of Cochno Dairy  
431 farm. Abundant spots of Ig (heavy and light chain) are seen which is characteristic of  
432 colostrum. Ig (immunoglobulin), CN (caseins), <sup>2</sup>-LG (beta lactoglobulin),  $\pm$ -LA (alpha  
433 lactalbumin), <sup>2</sup>-MG (beta-2 microglobulin)

434 **Figure 2B:** 2DE reducing gel of pooled (quarters) day 10 post-calving milk samples. On a  
435 pH 3-10 range strip. Less Ig spots are seen here compared to the colostrum samples 2DE. Ig  
436 (immunoglobulin), CN (caseins), <sup>o</sup>-CN (kappa caseins), <sup>2</sup>-LG (beta lactoglobulin),  $\pm$ -LA  
437 (alpha lactalbumin), <sup>2</sup>-MG (beta-2 microglobulin).

438 **Figure 3:** Hp concentrations in individual quarter milk samples from day 1 to day 10 post  
439 parturition (median, 25<sup>th</sup> & 75<sup>th</sup> percentile as boxes, 10<sup>th</sup> & 90<sup>th</sup> percentile as whiskers and  
440 outliers)

441 **Figure 4:** Hp concentration in quarter milk in 4 dairy cows showing fluctuations within the  
442 day 1 to day 10 post calving period.

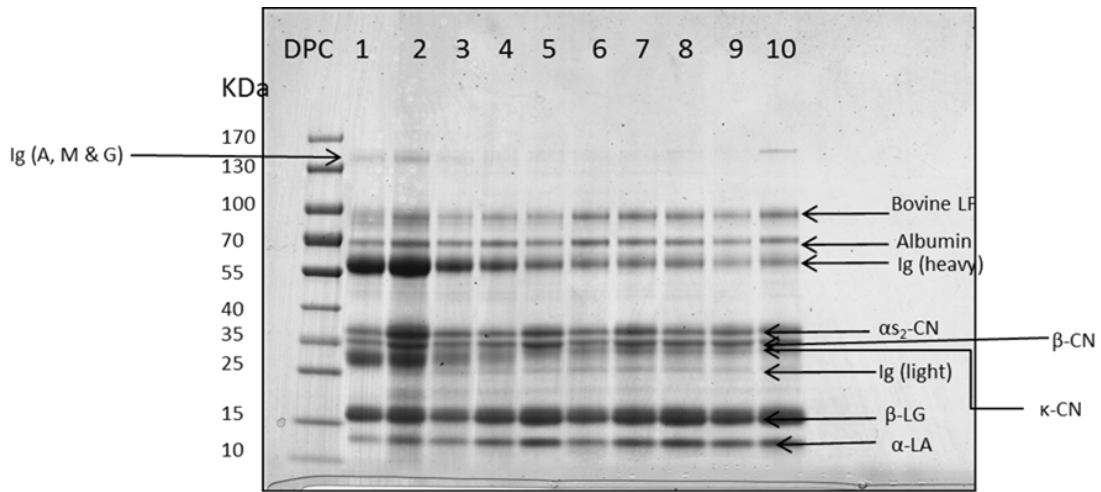
443 **Figure 5** Western blot on milk from day 1 to 10 post-calving stained with an IgG fraction of  
444 rabbit anti-bovine Hp conjugated to alkaline phosphate

445 **Figure 6:** Concentrations of daily Hp (median and range) from day 1-10 post-calving  
446 composite milk samples (n=22). Asterisks indicate significant differences from day 10 post  
447 calving by the Mann-Whitney test at \*P<0.05; \*\*P<0.01; \*\*\*P<0.001

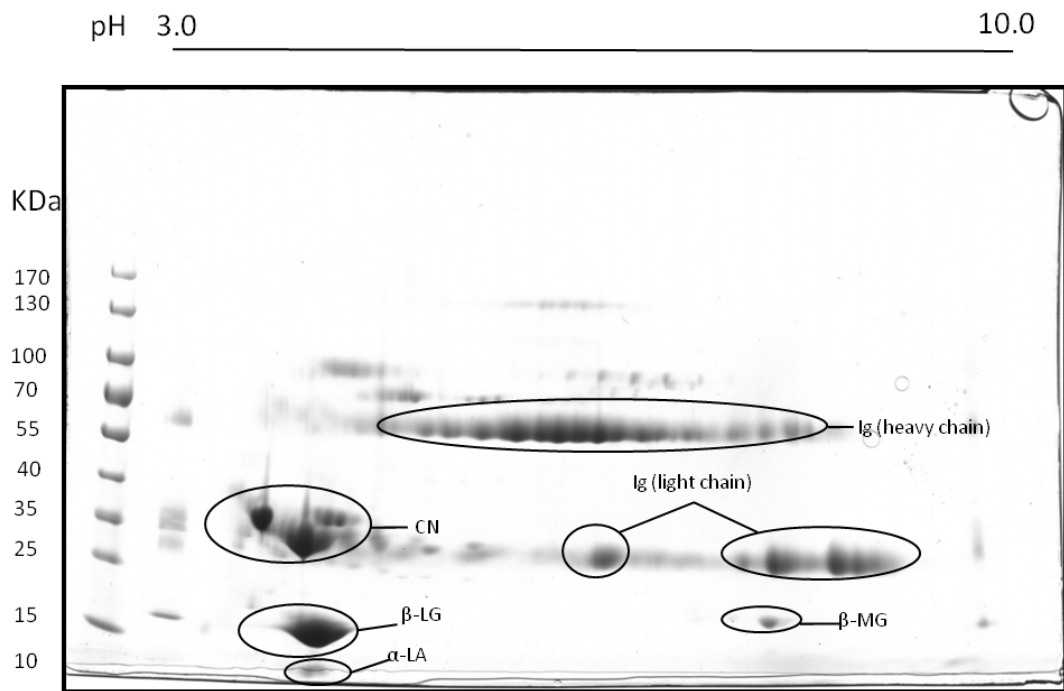
448 **Figure 7:** Concentrations of daily M-SAA3 (median and range) from day 1-10 post-calving  
449 composite milk samples (n=22). Asterisks indicate significant differences from day 10 post  
450 calving by the Mann-Whitney test at \*P<0.05; \*\*P<0.01; \*\*\*P<0.001

451 **Figure 8:** Concentrations of daily CRP (median and range) from day 1-10 post-calving  
452 composite milk samples (n=22). Asterisks indicate significant differences from day 10 post  
453 calving by the Mann-Whitney test at \*P<0.05; \*\*P<0.01; \*\*\*P<0.001

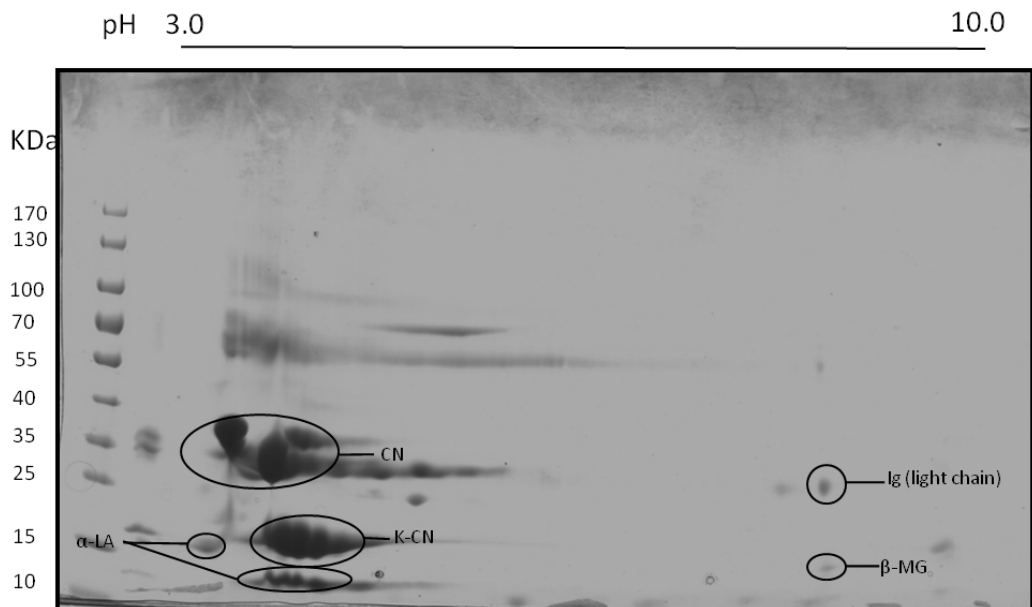
454 **Figure 1**



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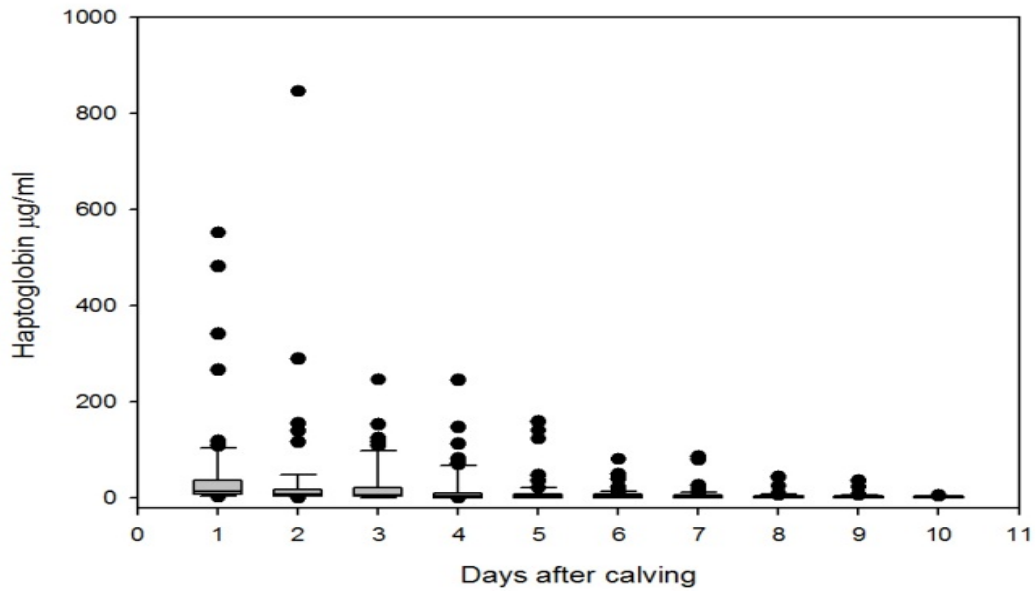
456 **Figure 2A**

457

458 **Figure 2B**

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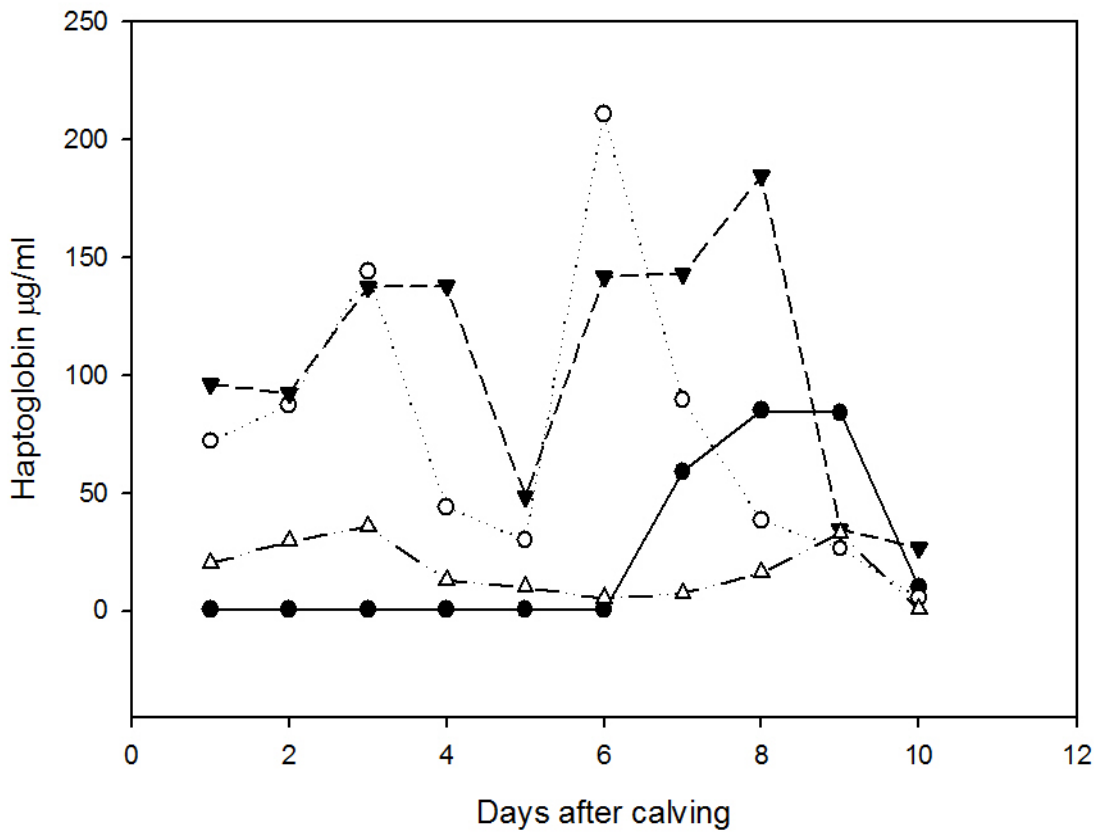
460 **Figure 3**



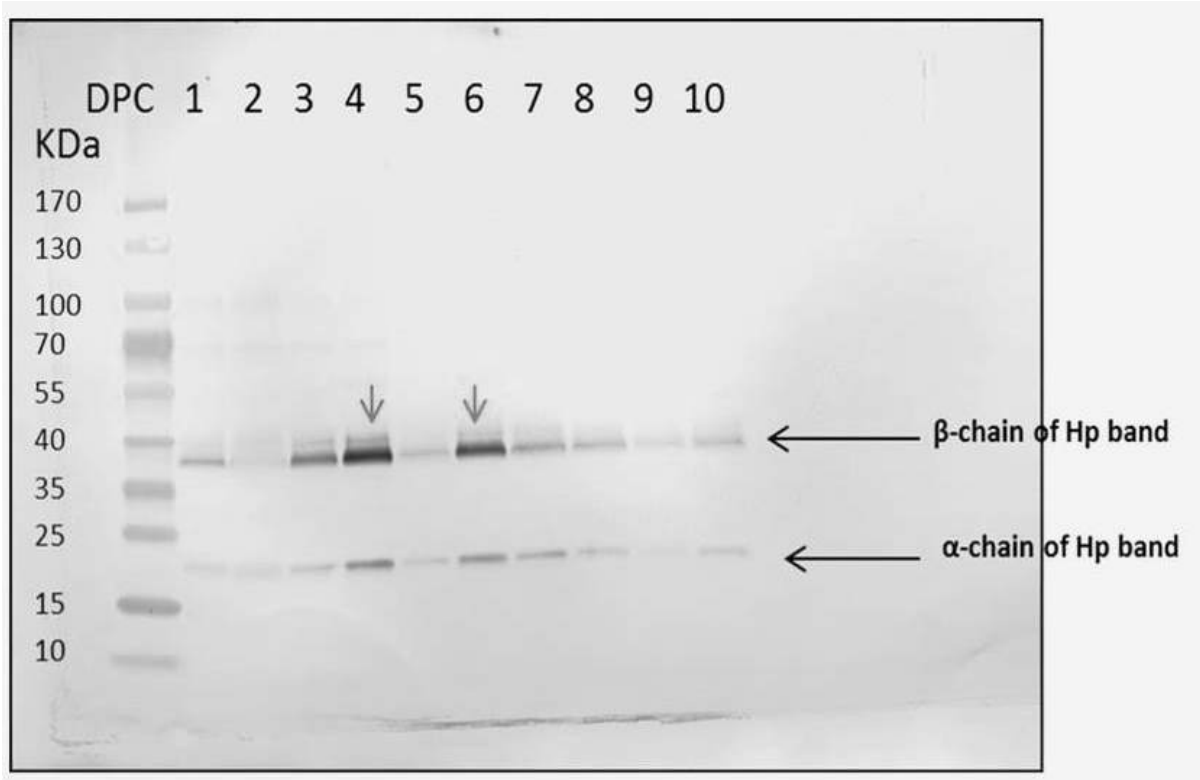
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463 **Figure 4**

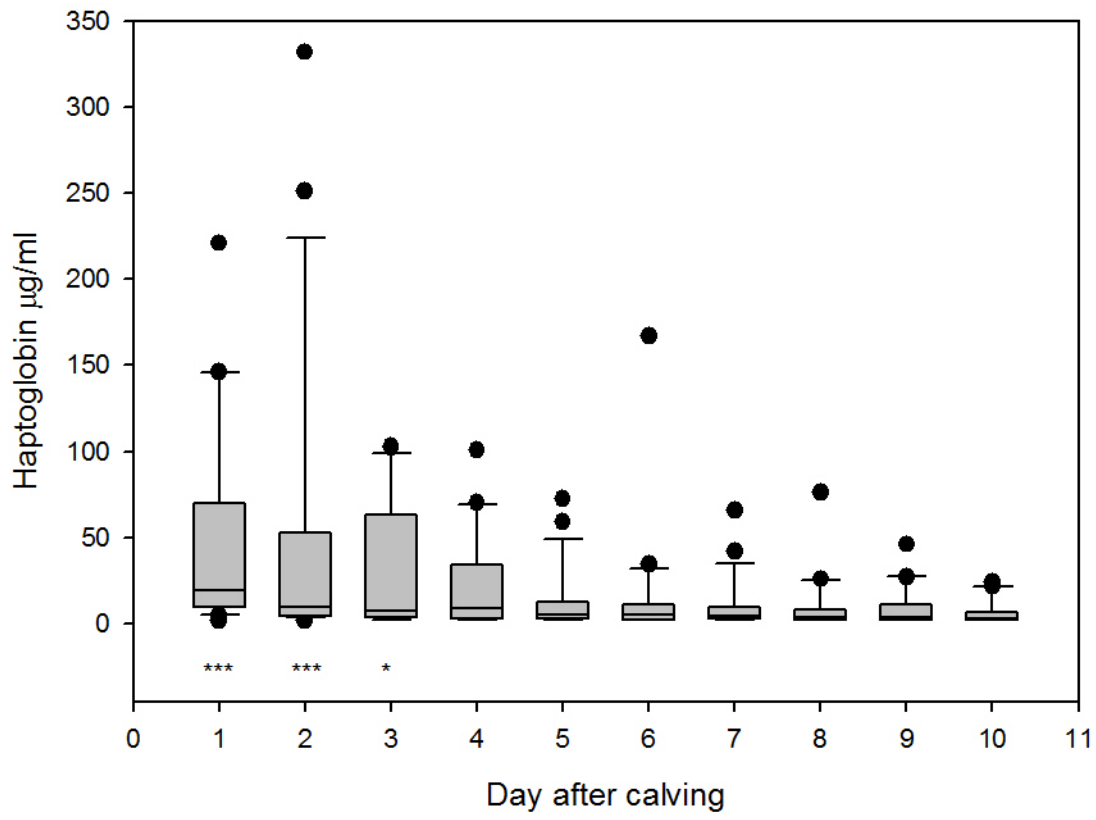
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465 **Figure 5**

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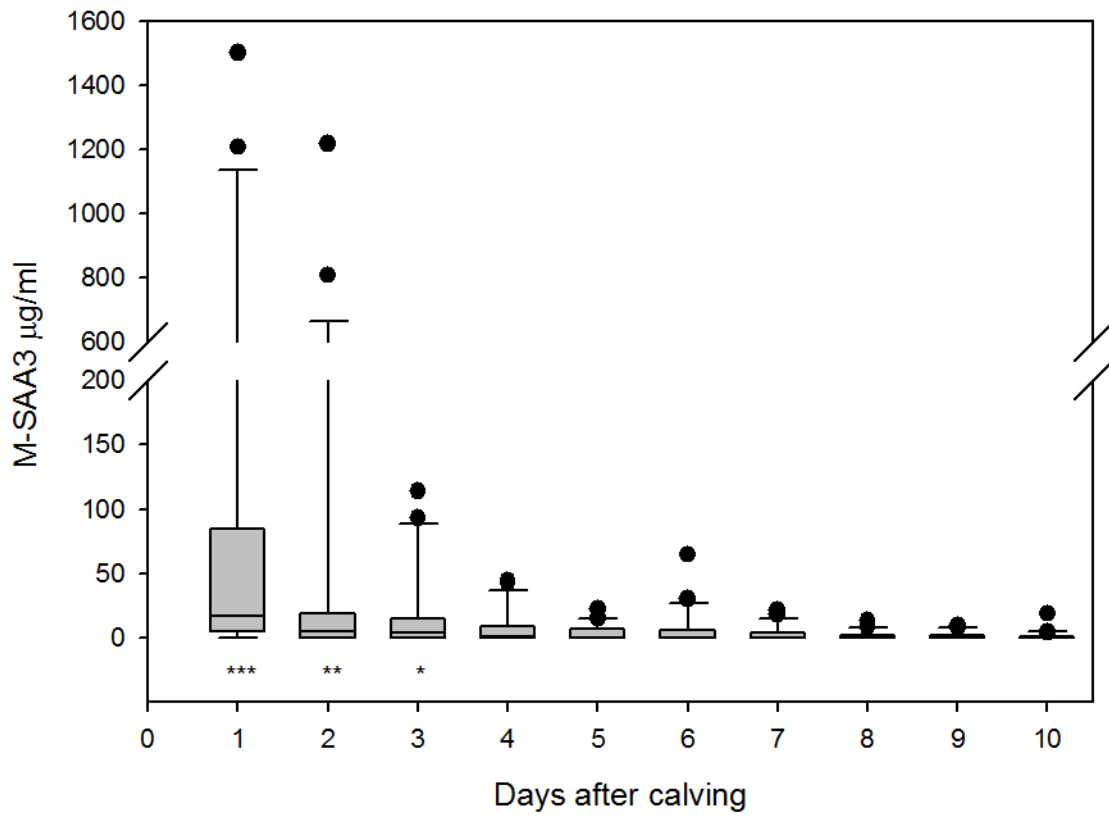
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468 **Figure 6**



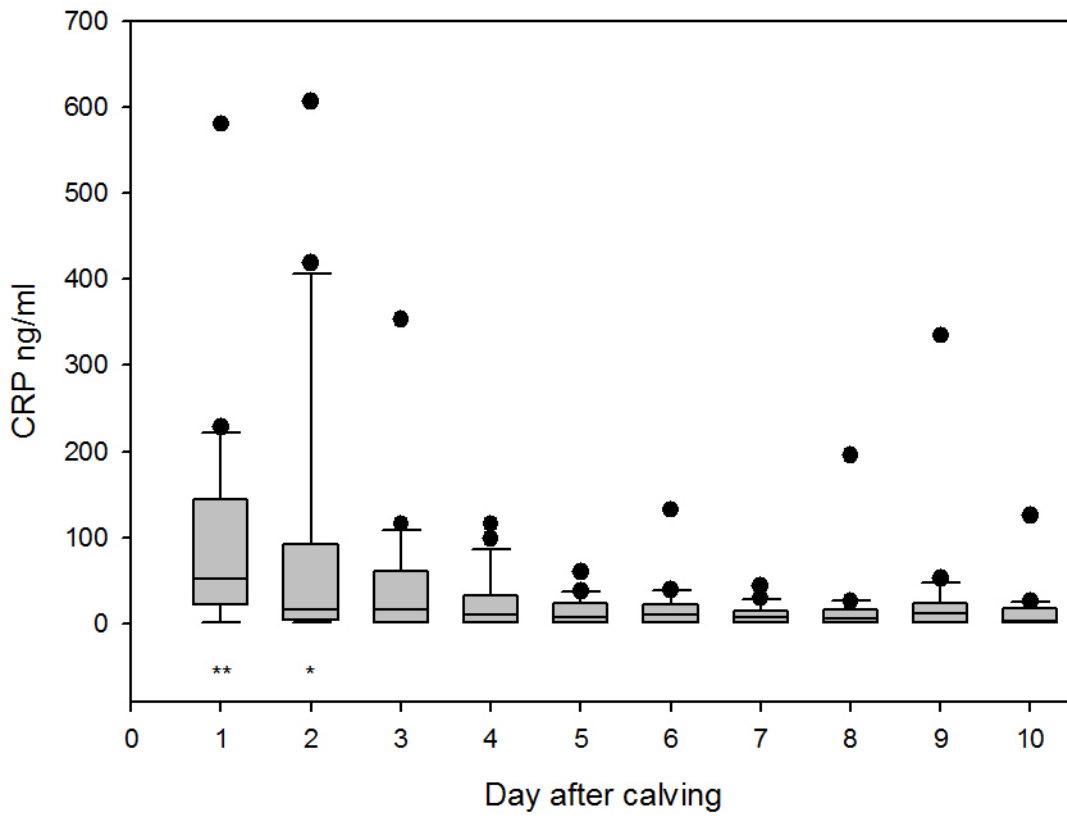
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470 **Figure 7**



471

472 **Figure 8**



473