



**HAL**  
open science

## Description of outcomes of experimental infection with feline haemoplasmas: copy numbers, haematology, Coombs' testing and blood glucose concentrations

Séverine Tasker, Iain R. Peters, Kostas Papasoulotis, Simon M. Cue, Barbara Willi, Regina Hofmann-Lehmann, Timothy J. Gruffydd-Jones, Toby G. Knowles, Michael J. Day, Chris R. Helps

### ► To cite this version:

Séverine Tasker, Iain R. Peters, Kostas Papasoulotis, Simon M. Cue, Barbara Willi, et al.. Description of outcomes of experimental infection with feline haemoplasmas: copy numbers, haematology, Coombs' testing and blood glucose concentrations. *Veterinary Microbiology*, 2009, 139 (3-4), pp.323. 10.1016/j.vetmic.2009.06.028 . hal-00526940

**HAL Id: hal-00526940**

**<https://hal.science/hal-00526940>**

Submitted on 17 Oct 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: Description of outcomes of experimental infection with feline haemoplasmas: copy numbers, haematology, Coombs' testing and blood glucose concentrations

Authors: Séverine Tasker, Iain R. Peters, Kostas Papasoulotis, Simon M. Cue, Barbara Willi, Regina Hofmann-Lehmann, Timothy J. Gruffydd-Jones, Toby G. Knowles, Michael J. Day, Chris R. Helps



PII: S0378-1135(09)00313-7  
DOI: doi:10.1016/j.vetmic.2009.06.028  
Reference: VETMIC 4483

To appear in: *VETMIC*

Received date: 22-11-2008  
Revised date: 10-6-2009  
Accepted date: 15-6-2009

Please cite this article as: Tasker, S., Peters, I.R., Papasoulotis, K., Cue, S.M., Willi, B., Hofmann-Lehmann, R., Gruffydd-Jones, T.J., Knowles, T.G., Day, M.J., Helps, C.R., Description of outcomes of experimental infection with feline haemoplasmas: copy numbers, haematology, Coombs' testing and blood glucose concentrations, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.06.028

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1        **Description of outcomes of experimental infection with feline haemoplasmas:**  
2        **copy numbers, haematology, Coombs' testing and blood glucose concentrations**

3

4        Séverine Tasker\*, Iain R. Peters, Kostas Pappasoulotis, Simon M. Cue, Barbara Willi<sup>1</sup>,  
5        Regina Hofmann-Lehmann<sup>1</sup>, Timothy J. Gruffydd-Jones, Toby G. Knowles, Michael  
6        J. Day and Chris R. Helps.

7

8        School of Clinical Veterinary Science, University of Bristol, Langford, Bristol, BS40  
9        5DU, United Kingdom

10        <sup>1</sup> Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

11

12        \*Corresponding author

13        School of Clinical Veterinary Science, University of Bristol, Langford, Bristol, BS40  
14        5DU, United Kingdom

15        Tel:    + 44 117 928 9558

16        Fax:    + 44 117 928 9559

17        E.mail: s.tasker@bristol.ac.uk

18

19

20 **Abstract**

21 The aim of this study was to compare blood copy, haematological and glucose values  
22 between cats experimentally infected with either *M. haemofelis* (Group HF: 10 cats),  
23 ‘*Candidatus M. haemominutum*’ (Group HM: 3 cats) or ‘*Candidatus M. turicensis*’  
24 (Group TU: 3 cats). Blood samples were collected regularly up to 85 days post-  
25 infection (DPI) for haemoplasma real-time quantitative PCR, haematology, Coombs’  
26 testing and blood glucose measurement. Statistical analysis was performed using a  
27 general linear model (ANOVA) appropriate for a repeated measures experiment with  
28 significance set as  $P < 0.05$ . Cats in Group TU had significantly lower blood copy  
29 numbers than cats in Group HF ( $P < 0.001$ ) and HM ( $P < 0.001$ ). All Group HF cats  
30 developed anaemia (often severe), macrocytosis and evidence of erythrocyte-bound  
31 antibodies whereas Groups HM and TU cats did not. Group HF had significantly  
32 lower PCVs, haemoglobin concentrations and red blood cell counts, and significantly  
33 higher mean cell volumes, than Groups HM and TU. In Group HF, erythrocyte-  
34 bound antibodies reactive at 4°C (both IgM and IgG) appeared between 8 and 22 DPI  
35 and persisted for two to four weeks, whereas those reactive at 37°C (primarily IgG)  
36 appeared between 22 and 29 DPI and persisted for one to five weeks. In most cats  
37 antibodies appeared after the fall in haemoglobin started. Although Group TU had  
38 significantly lower glucose concentrations than Groups HF ( $P = 0.006$ ) and HM  
39 ( $P = 0.027$ ), mean blood glucose concentrations remained within the reference range in  
40 all groups. This study demonstrates that *M. haemofelis* infection, in contrast to  
41 ‘*Candidatus M. haemominutum*’ and ‘*Candidatus M. turicensis*’ infection, can result  
42 in a severe macrocytic anaemia and the development of cold and warm reactive  
43 erythrocyte-bound antibodies.

44 **Keywords: haemoplasma; quantitative real-time PCR; coombs' test;**  
45 **autoagglutination; glucose**

46

## 47 **Introduction**

48 Three feline haemoplasma species are described; *Mycoplasma haemofelis*,  
49 '*Candidatus M. haemominutum*' and '*Candidatus M. turicensis*' (Foley and Pedersen,  
50 2001; Neimark et al., 2001, 2002; Willi et al., 2005; Willi et al., 2006). *M. haemofelis*  
51 infection often causes haemolysis (Berent et al., 1998; Foley et al., 1998; Westfall et  
52 al., 2001) but '*Candidatus M. haemominutum*' infection does not usually result in  
53 clinically significant anaemia (Foley et al., 1998; Westfall et al., 2001; Tasker et al.,  
54 2006a) without concurrent immunocompromisation (George et al., 2002).  
55 Experimental '*Candidatus M. turicensis*' has only been evaluated in one previous  
56 study in which two cats developed anaemia secondary to infection (Willi et al., 2005)  
57 with more severe anaemia developing in the one cat that had been  
58 immunocompromised before inoculation.

59 Positive Coombs' (direct antiglobulin) tests, indicating the presence of  
60 erythrocyte-bound antibodies, have been reported with haemoplasma infections in a  
61 number of species, including cats (Bundza et al., 1976; Harvey and Gaskin, 1978;  
62 Hoffmann et al., 1981). It has been proposed that such antibodies may contribute to  
63 the haemoplasma-associated haemolysis. However, investigations describing  
64 Coombs' tests in haemoplasma-infected cats have varied greatly with respect to the  
65 methods described, or used, to perform Coombs' testing (reagents, dilutions,  
66 temperatures employed and timing of sample collection) (Maede et al., 1974; Maede  
67 and Hata, 1975; Zulty and Kociba, 1990; Alleman et al., 1999; Willi et al., 2005).

68 Additionally, studies have not yet described Coombs' results in cats infected with  
69 each of the three feline haemoplasma species.

70 Severe hypoglycaemia has also been reported in several animal species acutely  
71 infected with haemoplasmas; sheep (*M. ovis*) (Sutton, 1977), an opossum  
72 ('*Candidatus M. haemodidelphidis*') (Messick et al., 2000), pigs (*M. suis*) (Zachary  
73 and Smith, 1985; Smith et al., 1990), llamas ('*Candidatus M. haemolamae*')  
74 (McLaughlin et al., 1990; Messick et al., 2002) and calves (*Haemobartonella bovis*)  
75 (Love et al., 1977). Hypoglycaemia was reported in one moribund cat infected with a  
76 feline haemoplasma species (Harvey and Gaskin, 1977) but further details were not  
77 presented. A recent prevalence study found no significant difference in blood glucose  
78 concentrations between naturally haemoplasma infected and non-haemoplasma  
79 infected cats (Willi et al., 2006), although differentiation between acute and chronic  
80 haemoplasma was not possible.

81 The aim of this study was to describe and compare blood copy numbers and  
82 haematological changes, including Coombs' test results, and blood glucose  
83 concentrations following experimental infection of cats with either *M. haemofelis*,  
84 '*Candidatus M. haemominutum*' or '*Candidatus M. turicensis*'.

## 85 **Materials and methods**

### 86 ***Study design and protocol***

87 Sixteen barrier-maintained specific pathogen (retroviral) free-derived domestic-  
88 shorthaired cats, aged 7 months, were used. Due to the variation in haemoplasma  
89 copy number anticipated with *M. haemofelis* infection (Tasker et al., 2003; Tasker et  
90 al., 2006b), and the undertaking of a parallel study investigating subsequent  
91 haemoplasma copy number variation in the blood and tissues of some of the same cats  
92 (Tasker et al., 2009), more cats were infected with *M. haemofelis* than the other two

93 species. Ten cats were randomly assigned to the *M. haemofelis* group; Group HF  
94 (cats HF1 to HF4, HF6 to HF10 and HF12; six entire females and four neutered  
95 males), three to the ‘*Candidatus M. haemominutum*’ group; Group HM (cats HM1,  
96 HM2 and HM4; one entire female and two neutered males), and three to the  
97 ‘*Candidatus M. turicensis*’ group; Group TU (cats TU1, TU2 and TU4; three neutered  
98 males). The discontinuous numbering of the cats was due to the inclusion of  
99 additional cats in the parallel study (Tasker et al., 2009) mentioned above.

100 Blood samples were collected on Day 0 of the study, immediately before  
101 haemoplasma inoculation. Blood was placed into EDTA-anticoagulant for complete  
102 blood count using a Cell Dyn 3700 analyser (Abbott, Illinois, USA), packed cell  
103 volume (PCV) determination (microhaematocrit tube centrifugation) and  
104 haemoplasma real-time quantitative PCR (qPCR), whilst a drop of remaining non-  
105 anticoagulated blood was used for blood glucose measurement (Accu-chek Aviva  
106 blood glucose system, Roche Diagnostics Ltd., Lewes, East Sussex, UK).

107 Experimental inoculation of all cats with the respective haemoplasma species was  
108 carried out using heparinised blood collected from three barrier-maintained carrier  
109 donor cats. Cat blood types were predetermined (RapidVet-H blood typing cards,  
110 DMS Laboratories Inc, New Jersey, US) to be compatible (all type A). Following  
111 collection, the heparinised blood was placed on wet ice and two mls of heparinised  
112 donor blood was given intravenously to each of the 16 cats, via pre-placed cephalic  
113 intravenous catheters, within 5 minutes of collection. The haemoplasma inoculum  
114 doses comprised  $1.55 \times 10^8$  *M. haemofelis* copies,  $5.04 \times 10^6$  ‘*Candidatus M.*  
115 *haemominutum*’ copies or  $2.42 \times 10^6$  ‘*Candidatus M. turicensis*’ copies per cat.

116 Blood samples were collected from all cats three times weekly from 2 to 85 days  
117 post-infection (DPI): twice weekly, 0.3 ml blood was used for PCV, haemoplasma

118 qPCR and blood glucose measurement, and once weekly, 1 ml blood was used for  
119 haematological examination, Coombs' testing, PCV, haemoplasma qPCR and blood  
120 glucose measurement.

121 Cats were monitored daily for ill health (dehydration, pallor, anorexia) and were  
122 given subcutaneous fluid therapy (lactated Ringer's, up to 100 ml/kg/day) if they were  
123 pale, dehydrated or anorexic. Occasionally PCV was measured more frequently in  
124 individual cats if additional monitoring was needed to dictate therapy (data not  
125 shown). If PCV fell  $\leq 10\%$ , doxycycline at 10mg/kg PO (followed by 3 mls water by  
126 syringe PO) was given daily until the PCV rose  $> 10\%$ .

127 All procedures and experiments described were undertaken under a project license  
128 approved under the UK Animals (Scientific Procedures) Act 1986.

#### 129 ***DNA extraction***

130 DNA was extracted from 100  $\mu$ l EDTA-anticoagulated blood (Macherey-Nagel  
131 Nucleospin Blood kit, ABgene, Epsom, UK), eluting into 100  $\mu$ l Buffer BE. For each  
132 batch of samples, blood samples from known haemoplasma non-infected and infected  
133 cats were subjected to DNA extraction as negative and positive controls respectively.

#### 134 ***PCR assays***

135 Haemoplasma qPCRs were carried out as previously described (Peters et al.,  
136 2008), using five  $\mu$ l of DNA template per 25  $\mu$ l reaction. The assays consist of three  
137 distinct duplex qPCRs in which there is simultaneous detection of one of the  
138 haemoplasma species (*M. haemofelis*, '*Candidatus M. haemominutum*' or  
139 '*Candidatus M. turicensis*') and feline 28S rDNA as an internal control. DNA  
140 extraction and PCR analyses were performed on the days of blood collection. DNA  
141 samples from known haemoplasma-infected cats and water were subjected to qPCR as  
142 positive and negative controls respectively. Haemoplasma copy number



143 quantification was derived by comparison to a standard curve generated by  
144 amplification of plasmids containing cloned 16S rDNA PCR products from each of  
145 the haemoplasma species (Tasker et al., 2003; Willi et al., 2005; Peters et al., 2008).

#### 146 *Coombs' testing*

147 Coombs' testing was performed within 24 hours of blood collection. An aliquot  
148 of EDTA-blood was suspended in an excess volume of cold phosphate buffered saline  
149 (PBS) (pH 7.4) and centrifuged (2200 g, 3 min). The supernatant and buffy coat were  
150 removed before resuspending the pelleted red blood cells (RBCs) in PBS and  
151 repeating the centrifugation and removal of supernatant and buffy coat twice to wash  
152 the RBCs a total of three times. A 2.5% suspension of the packed, washed RBCs was  
153 then made in PBS. Serial dilutions (1:5 to 1:640) in PBS of polyvalent feline  
154 Coombs' reagent (antiserum which reacts with feline IgG, IgM, IgA and C3; ICN  
155 Flow, Basingstoke, UK), anti-feline IgG Fc and anti-feline IgM Fc antiserum (both  
156 Nordic Laboratories, Tilburg, The Netherlands) were made (25  $\mu$ l total) in two U-  
157 bottomed 96 well plates. Antisera were pre-absorbed with a pool of normal feline  
158 RBCs. Four control wells with PBS alone were also set up as negative controls. To  
159 each well, 25  $\mu$ l of the 2.5% RBC suspension were added and mixed by tapping the  
160 plate. The two plates were then incubated for one hour; one at 4°C and one at 37°C.  
161 The plates were examined for agglutination, with the recorded titre being the inverse  
162 of the reagent dilution in the last well where unequivocal agglutination occurred.  
163 Positive titres were described as being low if  $\leq 80$ , moderate if 160 to 320 and high if  
164  $\geq 640$ . The titre, thermal activity and deduced antibody class of any positive cases  
165 were recorded. If agglutination was present in the PBS control wells, the titre and  
166 class of the antibody involved could not be recorded in the Coombs' test and these  
167 cases were defined as having persistent autoagglutination, due to the presence of

168 erythrocyte-bound antibody despite RBC washing, at the temperature at which it was  
169 demonstrated. Cats were described as having cold-reactive antibodies if persistent  
170 agglutination or Coombs' positivity was only present or stronger at 4°C compared to  
171 37°C, whereas those in which persistent agglutination or Coombs' positivity was only  
172 present or stronger at 37°C compared to 4°C were said to have warm-reactive  
173 antibodies (Gehrs and Friedberg, 2002). Cats with equal persistent agglutination or  
174 Coombs' positive reactions at both 4°C and 37°C were described as having both  
175 warm and cold-reactive antibodies (Gehrs and Friedberg, 2002).

### 176 ***Analysis of results***

177 Data were explored by plotting haematological variables, blood glucose  
178 concentrations and haemoplasma copy numbers for each cat against time, and mean  
179 values of these variables for each group (HF, HM or TU) against time. The Coombs'  
180 test results were tabulated, and summary Coombs' results plotted against time  
181 alongside haemoglobin values to discern any pattern of Coombs' reactivity in relation  
182 to onset of anaemia.

183 To evaluate for any effect of different haemoplasma species infection on  
184 haemoplasma copy number, haematological and blood glucose data, these variables  
185 were compared between each group (HF, HM or TU) using a general linear model  
186 (ANOVA) appropriate for a repeated measures experiment. The haemoplasma copy  
187 numbers were logged to allow a more biologically meaningful view of the variation in  
188 copy numbers recorded over time. Using these analyses, when a statistically  
189 significant difference was identified between the groups, the nature of the difference  
190 between individual groups was determined using the Tukey *post hoc* multiple  
191 comparisons test for normally distributed data. The residuals from this model were

192 checked to ensure that the assumptions necessary for this parametric analysis were  
193 met.

194 When exploration of the data showed a fall in PCV in all three groups between  
195 Days 0 and 20 pi, to retrospectively evaluate whether haemoplasma infection with  
196 each of the species was associated with a significant change in PCV, a repeated  
197 measures ANOVA test was performed for all three groups from Day 0 to 20 pi. This  
198 test was applied retrospectively, after inspection of the data, and so the results can be  
199 viewed as a guide only.

200 Statistical analysis was performed using Statistical Package for Social Scientists  
201 (SPSS) version 14. Significance was taken at a P value of  $< 0.05$ .

## 202 **Results**

### 203 *Haemoplasma copy numbers*

204 Figures 1A and 1B show the blood haemoplasma copy numbers recorded in  
205 Groups HF, HM and TU over time. All cats were haemoplasma PCR negative on Day  
206 0 and became positive by 5 DPI. Some marked variation in *M. haemofelis* copy  
207 number was evident over time in each of the HF cats, especially until around 20 DPI,  
208 which was not evident in the other two infection groups other than mild variation in  
209 one of the TU group cats between 22 and 30 DPI. Mean maximum copy number was  
210 reached on 15 DPI in both the HF ( $\sim 4.4 \times 10^9$  copies/ml blood) and TU groups ( $\sim 4.2$   
211  $\times 10^6$  copies/ml blood) but on 29 DPI in the HM group ( $\sim 2.5 \times 10^9$  copies/ml blood).  
212 A repeated measures ANOVA showed that there was a highly significant ( $P < 0.001$ )  
213 day by group interaction on blood copy numbers. No significant difference ( $P=0.936$ )  
214 in copy numbers between the HF and HM groups was found but the TU group had  
215 significantly lower copy numbers than both Groups HF ( $P<0.001$ ) and HM ( $P<0.001$ ).

216 ***Haematology***

217 Figure 2 depicts haematological values recorded for all groups. All cats in Group  
218 HF group became anaemic (PCV < 25%), being severe (PCV < 15%) in 8 of the 10  
219 cats with the mean lowest PCV value recorded on 15 DPI. None of the Group HM or  
220 TU cats became anaemic. A repeated measures ANOVA showed that there was a  
221 highly significant ( $P < 0.001$ ) day by group interaction on PCV values. No significant  
222 difference ( $P=0.319$ ) in PCV was found between the HM and TU groups but Group  
223 HF had significantly lower PCVs than both Groups HM ( $P=0.018$ ) and TU ( $P=0.001$ ).  
224 Haemoglobin (Hb) concentrations and red blood cell counts (RBCC) displayed  
225 similar changes and statistical results to those seen with PCV. Mean cell volume  
226 (MCV) remained within the reference range in all Group HM and TU cats but rose  
227 above normal in all Group HF cats. A repeated measures ANOVA showed that there  
228 was a highly significant ( $P<0.001$ ) day by group interaction on MCV values. No  
229 significant difference ( $P=0.614$ ) in MCV between Groups HM and TU was found but  
230 the Group HF had significantly higher MCVs than both Group HM ( $P<0.001$ ) and TU  
231 ( $P<0.001$ ). The white blood cell counts were not significantly different between the  
232 groups ( $P=0.306$ ).

233 A repeated measures ANOVA test performed in each group from 0 to 20 DPI  
234 found that all three haemoplasma species were associated with a significant PCV  
235 decrease over this time period:  $P<0.001$  for Groups HF and HM, and  $P=0.001$  for  
236 Group TU.

237 ***Coombs' test***

238 All cats were Coombs' test negative on Day 0. Group HM and TU cats remained  
239 Coombs' negative throughout the study whereas all cats in Group HF showed positive  
240 results at various time points. Those cats that showed persistent autoagglutination in

241 the PBS control wells at 4°C and/or 37°C also showed agglutination in all test wells at  
242 that temperature, which precluded determination of the titre and class of the antibody  
243 involved at that temperature. Table 1 shows Coombs' results from 0 to 64 DPI for all  
244 Group HF cats. Cold-reactive antibodies were seen at the majority of time points,  
245 whilst a moderate number of time points showed both cold and warm-reactive  
246 antibodies and a few showed warm-reactive antibodies.

247 Regarding kinetics of the antibody response, erythrocyte-bound antibodies  
248 reactive at 4°C appeared first, starting in all cats between 8 and 22 DPI and lasting for  
249 two to four weeks. Erythrocyte-bound antibodies reactive at 37°C tended to appear  
250 slightly later, starting in all cats between 22 and 29 DPI and lasting for one to five  
251 weeks. Figure 3 shows that in a few cats antibodies reactive at 4°C appeared when  
252 the fall in Hb first became marked, whereas in the majority of cats they appeared at  
253 the nadir in Hb concentration. Antibodies reactive at 37°C first appeared at the nadir  
254 in Hb concentration and were present in most cats as the Hb values increased again.

### 255 ***Blood glucose concentrations***

256 All three group mean glucose values remained within the reference range (Figure  
257 4), however a repeated measures ANOVA showed that there was a significant  
258 ( $P=0.011$ ) day by group interaction on blood glucose values. No significant  
259 difference ( $P=0.997$ ) in blood glucose concentrations between Groups HF and HM  
260 was found but Group TU had significantly lower blood glucose concentrations than  
261 both Groups HF ( $P=0.006$ ) and HM ( $P=0.027$ ).

### 262 ***Treatment***

263 None of the Group HM or TU cats required treatment. Group HF cats were given  
264 subcutaneous fluid therapy due to dehydration, anorexia and/or pallor (HF1 on 8, 10,  
265 12, 13, 14 and 15 DPI, HF2 on 13, 14, 15, 20 and 25 DPI, HF3 on 14 DPI, HF6 on 8,

266 10, 13, 14, 15 and 24 DPI, HF8 on 14 and 15 DPI, HF9 on 8, 10 and 13 DPI, HF9 on  
267 8, 10 and 13 DPI, HF10 on 8 and 16 DPI, and HF12 on 10 and 13 DPI). Doxycycline  
268 was administered when PCV fell to  $\leq 10\%$  (HF1 on 14, 15, 17 and 20 DPI, HF2 on 14  
269 DPI only, HF3 on 19, 20 and 22 DPI, HF6 on 15 and 24 DPI, HF8 on 20 DPI and  
270 HF9 on 15 DPI). HF6 was given doxycycline in error (PCV of 11%) on 24 DPI.

## 271 **Discussion**

272 This study is the first to describe contemporaneous experimental infection with  
273 either *M. haemofelis*, '*Candidatus M. haemominutum*' or '*Candidatus M. turicensis*'  
274 in cats of similar signalment maintained under identical conditions.

275 Group TU had significantly lower blood copy numbers than the HF and HM  
276 groups. The phenomenon of lower copy numbers with '*Candidatus M. turicensis*'  
277 infection has previously been reported in naturally infected cats (Willi et al., 2006).  
278 Although the inoculum dose used for Group TU was far lower than for Group HF, it  
279 was only about half that used for Group HM, yet the maximal copy numbers reached  
280 in Groups TU and HM differed by several logs. Thus the lower TU group copy  
281 numbers are not likely due to inoculum dose alone. The HF and HM group copy  
282 numbers remained high for most of the study period whereas those of the TU group  
283 fell markedly after 38 DPI, and were negative in all TU cats by 45 DPI. These  
284 negative qPCR results may not have represented clearance of infection in all cats  
285 since TU2 and TU4 both generated positive qPCR results, albeit at very low copy  
286 numbers, later in the course of infection. However, these data suggest that  
287 spontaneous elimination of '*Candidatus M. turicensis*' infection may be possible  
288 without treatment, as has been reported previously in two '*Candidatus M. turicensis*'  
289 experimentally infected cats (Willi et al., 2005), although this was late in the course of  
290 infection in one cat (100 DPI).

291 The three feline haemoplasma species had differing pathogenicities. All 10 HF  
292 group cats had PCVs of < 20% during the study, with seven and four cats reaching  
293 PCVs of < 15% and < 10% respectively. This is in contrast to a previous  
294 experimental *M. haemofelis* study using the same isolate (Tasker et al., 2006b) in  
295 which the induced anaemia was less severe. The inoculum dose used in the previous  
296 study (Tasker et al., 2006b) was not determined, although maximum *M. haemofelis*  
297 copy numbers in the cats of that and the current study were within one log of each  
298 other. Both studies comprised a mixture of neutered male and entire female cats, so  
299 gender differences are unlikely to be responsible. The cats in the current study were  
300 all 7 months of age, younger than the 5 to 8 year old cats in the previous study (Tasker  
301 et al., 2006b). Age may play a part in the severity of *M. haemofelis*-induced anaemia,  
302 possibly due to immunological immaturity in younger animals (Day, 2007). Indeed,  
303 one study of naturally infected cats (Sykes et al., 2008) suggested that younger  
304 animals may be predisposed to clinical haemoplasmosis due to *M. haemofelis*.

305 Although ‘*Candidatus M. haemominutum*’ and ‘*Candidatus M. turicensis*’ were  
306 not associated with induction of anaemia or clinical signs, both were still associated  
307 with a significant drop in PCV up to 20 DPI, indicating an effect on RBC parameters,  
308 albeit not severe. Anaemia has been reported in two 10 year old male neutered cats  
309 (Willi et al., 2005) following intravenous inoculation of the same isolate of  
310 ‘*Candidatus M. turicensis*’ that was used in the current study. However the fall in  
311 PCV was relatively mild in both cats with the more severe anaemia occurring in the  
312 cat given steroids prior to inoculation.

313 Group HF had significantly elevated MCVs, believed to reflect the presence of  
314 larger immature RBCs due to a regenerative response, although reticulocyte counts  
315 were not performed. A recent study reported that cats naturally infected with *M.*

316 *haemofelis* had significantly elevated MCVs (Sykes et al., 2008), although concurrent  
317 feline leukaemia virus infection could have played a part in the development of  
318 macrocytosis in the cats of that study.

319 This study is the first to comprehensively describe Coombs' testing during the  
320 course of experimental feline haemoplasmosis. Only HF cats showed positive results.  
321 Serum biochemistry was not performed routinely due to the need to restrict the blood  
322 volume collection but limited analysis (data not shown), performed between 22 and  
323 36 DPI in the HF cats to monitor treatment, revealed hyperbilirubinaemia on 22  
324 and/or 29 DPI in all but two Group HF cats. This hyperbilirubinaemia likely reflects  
325 haemolysis and was present when erythrocyte-bound antibodies were prominent.

326 All developed cold-reactive antibodies, consistent with the presence of IgM or  
327 IgG antibodies that fully or partially elute from the surface of the RBCs at  
328 physiological temperatures *in vitro*. Cold-reactive erythrocyte-bound antibodies have  
329 been reported previously with haemoplasma infection in cats (Zulty and Kociba,  
330 1990), dogs (Bundza et al., 1976; Bellamy et al., 1978), and pigs (Hoffmann et al.,  
331 1981; Zachary and Smith, 1985), although the descriptions of the cold agglutinins and  
332 the method of detection used was inconsistent. Their significance is not known  
333 although some have suggested that they are involved in haemoplasma-induced  
334 anaemia (Zulty and Kociba, 1990). In this study the clear appearance and  
335 disappearance of such antibodies during *M. haemofelis*-associated anaemia suggests  
336 that infection induced the antibodies but it is difficult to assign a definitive  
337 pathophysiological role to the antibodies since the temperature at which the *in vitro*  
338 cold agglutination is observed does not reflect normal body temperature. However  
339 IgM is very efficient at complement fixation via the classical pathway, so it is possible  
340 that complement deposition onto RBCs occurs at the lower temperatures of the



341 peripheral circulation (28°C to 31°C; (Gehrs and Friedberg, 2002)), even if the  
342 initiating antibodies then dissociate from RBCs at core body temperature. In humans,  
343 cold agglutinins may have thermal activity up to 30°C (Rosse and Adams, 1980) and  
344 it is said that pathological cold agglutinins are characterised by having activity at such  
345 a temperature range (Gehrs and Friedberg, 2002). Although not reported here, we  
346 also evaluated the thermal reactivity of these antibodies by a further incubation at  
347 room temperature (18 to 20°C), but the results of these tests mirrored those performed  
348 at 37°C rather than 4°C, suggesting that the cold agglutinins identified in our study did  
349 not have a wide thermal activity.

350 In three cats classical warm-reactive antibodies alone were identified at certain  
351 time points, but a larger number (eight) showed evidence of both warm and cold-  
352 reactive antibodies. Warm-reactive antibodies tended to appear after the cold-reactive  
353 antibodies, once the anemia was established. Warm-reactive erythrocyte-bound  
354 antibodies have been previously demonstrated in haemoplasma-infected cats (Zulty  
355 and Kociba, 1990; Alleman et al., 1999) but descriptions of the nature of the warm  
356 antibodies and the method of detection used was inconsistent in those studies.

357 The role of warm-reactive antibodies in the pathogenesis of anaemia is less  
358 contentious, since these are active at normal body temperature, although they tended  
359 to appear after the anaemia had developed. Although cold-reactive antibodies  
360 appeared earlier in the course of infection, they were still not present at the time that  
361 Hb values started to fall. We cannot rule out that RBC bound antibodies were present  
362 before the onset of Coombs' positive test results but that they were undetectable due  
363 to the sensitivity of the Coombs' test being too low during early infection.  
364 Alternatively, it is possible that the erythrocyte antibodies arose merely as a sequel to  
365 *M. haemofelis* infection and/or haemolysis rather than initiating the anaemia. Binding

366 of haemoplasma organisms to RBCs could unmask previously ‘cryptic’ RBC antigens  
367 and trigger the *de novo* induction of erythrocyte autoantibodies. The observed  
368 antibodies could also be haemoplasma-specific and be RBC-associated by virtue of  
369 the fact that the pathogen resides on the RBC surface. Previous studies have  
370 demonstrated that serum from haemoplasma-infected cats is able to agglutinate  
371 autologous parasitized RBCs and neuraminidase-treated non-parasitised RBCs but not  
372 normal RBCs (Zulty and Kociba, 1990), suggesting that cryptic RBC antigens may  
373 well be targeted by autoantibodies. Haemolysis itself may also expose such cryptic  
374 antigens.

375       Blood glucose concentrations in experimentally haemoplasma-infected cats  
376 have not previously been reported, other than a non-descriptive reference to a single  
377 case (Harvey and Gaskin, 1977), and two experimentally ‘*Candidatus M. turicensis*’-  
378 infected cats (Willi et al., 2005) in which no significant hypoglycaemia was seen  
379 although one cat, that had been immunosuppressed before inoculation, had glucose  
380 levels at the lower limit of the reference range but only starting after peak bacteraemia  
381 was recorded<sup>1</sup>. Interestingly the current study found that Group TU cats had  
382 significantly lower blood glucose concentrations than the other two groups, although  
383 mean values remained within the reference range and no clinical effect was seen.  
384 Hypoglycaemia has been reported in other species (Love et al., 1977; Sutton, 1977;  
385 Zachary and Smith, 1985; McLaughlin et al., 1990; Smith et al., 1990; Messick et al.,  
386 2000; Messick et al., 2002), with the severity of hypoglycaemia being proportional to  
387 the degree of haemoplasma bacteraemia (McLaughlin et al., 1990). The reason for  
388 hypoglycaemia has not been fully elucidated although increased glucose utilisation  
389 has been demonstrated in *M. ovis*-infected RBCs (Sutton, 1976) and *M. suis* studies

---

<sup>1</sup> Personal communication BW, RHL

390 have suggested that this is due to the haemoplasma organisms themselves rather than  
391 the RBCs (Nonaka et al., 1996). Interestingly, many of the studies describing  
392 haemoplasma-associated hypoglycaemia fail to describe clinical signs consistent with  
393 significant hypoglycaemia (Zachary and Smith, 1985; McLaughlin et al., 1990; Smith  
394 et al., 1990; Messick et al., 2000), and two studies specifically describe marked  
395 hypoglycaemia in the absence of any associated clinical signs (Sutton, 1977;  
396 Burkhard and Garry, 2004). One of these studies (Burkhard and Garry, 2004)  
397 demonstrated the hypoglycaemia to be due to *in vitro* consumption by *M. ovis* since  
398 blood glucose measured immediately after collection from an infected lamb was  
399 normal, whereas the same blood submitted and ‘processed quickly’ (further details not  
400 given) revealed marked hypoglycaemia. The use of a handheld glucometer to  
401 measure blood glucose concentrations immediately in the current study may therefore  
402 have contributed to the observed normoglycaemia.

#### 403 **Conclusion**

404 This study demonstrates lower ‘*Candidatus M. turicensis*’ blood copy numbers in  
405 cats infected with this species compared with the other two species. Only *M.*  
406 *haemofelis*-infected cats developed a macrocytic anaemia, and evidence of  
407 erythrocyte-bound antibodies which appeared after the onset of decreasing RBC  
408 parameters, suggesting that they did not directly mediate the anaemia.  
409 Hypoglycaemia was not found in this study.

#### 410 **Acknowledgements**

411 This study was funded by the Wellcome Trust (Grant no 077718). R.H-L is  
412 the recipient of a professorship by the Swiss National Science Foundation (PP00B-  
413 102866 and PP00P3-119136). The authors wish to thank Suzanne Rudd and Mrs  
414 Sheila Jones for valued assistance with the study.

415 **References**

- 416 Alleman, A.R., Pate, M.G., Harvey, J.W., Gaskin, J.M., Barbet, A.F., 1999, Western  
417 immunoblot analysis of the antigens of *Haemobartonella felis* with sera from  
418 experimentally infected cats. J Clin Microbiol 37, 1474-1479.
- 419 Bellamy, J.E., MacWilliams, P.S., Searcy, G.P., 1978, Cold-agglutinin hemolytic  
420 anaemia and *Haemobartonella canis* infection in a dog. J Am Vet Med Assoc 173,  
421 397-401.
- 422 Berent, L.M., Messick, J.B., Cooper, S.K., 1998, Detection of *Haemobartonella felis*  
423 in cats with experimentally induced acute and chronic infections, using a polymerase  
424 chain reaction assay. Am J Vet Res 59, 1215-1220.
- 425 Bundza, A., Lumsden, J.H., McSherry, B.J., Valli, V.E., Jazen, E.A., 1976,  
426 Haemobartonellosis in a dog in association with Coombs' positive anaemia. Can Vet J  
427 17, 267-270.
- 428 Burkhard, M.J., Garry, F., 2004, Artfactual hypoglycemia associated with  
429 hemotropic mycoplasma infection in a lamb. Vet Clin Pathol 33, 244-248.
- 430 Day, M.J., 2007, Immune system development in the dog and cat. J Comp Pathol 137  
431 Suppl 1, S10-15.
- 432 Foley, J.E., Harrus, S., Poland, A., Chomel, B., Pedersen, N.C., 1998, Molecular,  
433 clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in  
434 domestic cats. Am J Vet Res 59, 1581-1588.
- 435 Foley, J.E., Pedersen, N.C., 2001, '*Candidatus* Mycoplasma haemominutum', a low-  
436 virulence epierythrocytic parasite of cats. Int J Syst Evol Microbiol 51, 815-817.
- 437 Gehrs, B.C., Friedberg, R.C., 2002, Autoimmune hemolytic anemia. Am J Hematol  
438 69, 258-271.

- 439 George, J.W., Rideout, B.A., Griffey, S.M., Pedersen, N.C., 2002, Effect of  
440 preexisting FeLV infection or FeLV and feline immunodeficiency virus coinfection  
441 on pathogenicity of the small variant of *Haemobartonella felis* in cats. *Am J Vet Res*  
442 63, 1172-1178.
- 443 Harvey, J.W., Gaskin, J.M., 1977, Experimental feline haemobartonellosis. *J Am*  
444 *Anim Hosp Assoc* 13, 28-38.
- 445 Harvey, J.W., Gaskin, J.M., 1978. Feline haemobartonellosis. In: 45th Annual  
446 Meeting of the American Animal Hospital Association, Salt Lake City, pp. 117-123.
- 447 Hoffmann, R., Schmid, D.O., Hoffmann-Fezer, G., 1981, Erythrocyte antibodies in  
448 porcine eperythrozoonosis. *Vet Immunol Immunopathol* 2, 111-119.
- 449 Love, J.N., Wilson, R.P., McEwen, E.G., Wiygul, G., 1977, Metabolism of  
450 [14C]glucose in *Haemobartonella*-like infected erythrocytes in splenectomized calves.  
451 *Am J Vet Res* 38, 739-741.
- 452 Maede, Y., Hata, R., 1975, Studies on feline haemobartonellosis. II. The mechanism  
453 of anemia produced by infection with *Haemobartonella felis*. *Jap J Vet Sci* 37, 49-54.
- 454 Maede, Y., Hata, R., Shibata, H., Niiyama, M., Too, K., Sonoda, M., 1974, Clinical  
455 observation on six cases of feline infectious anaemia. *J Jap Vet Med Assoc* 27, 267-  
456 272.
- 457 McLaughlin, B.G., Evans, C.N., McLaughlin, P.S., Johnson, L.W., Smith, A.R.,  
458 Zachary, J.F., 1990, An *Eperythrozoon*-like parasite in llamas. *J Am Vet Med Assoc*  
459 197, 1170-1175.
- 460 Messick, J.B., Berent, L.M., Ehrhart, E.J., Wasmer, C.C., 2000, Light and electron  
461 microscopic features of *Eperythrozoon*-like parasites in a North American Opossum  
462 (*Didelphis virginiana*). *J Zoo Wildl Med* 31, 240-243.

- 463 Messick, J.B., Walker, P.G., Raphael, W., Berent, L.M., Shi, X., 2002, '*Candidatus*  
464 *Mycoplasma haemodidelphidis*' sp. nov., '*Candidatus* *Mycoplasma haemolamae*' sp.  
465 nov and *Mycoplasma haemocanis* comb. nov., haemotrophic parasites from a  
466 naturally infected opossum (*Didelphis virginiana*), alpaca (*Lama pacos*) and dog  
467 (*Canis familiaris*): phylogenetic and secondary structural relatedness of their 16S  
468 rRNA genes to other mycoplasmas. *Int J Syst Evol Microbiol* 52, 693-698.
- 469 Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 2001, Proposal to transfer  
470 some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus  
471 *Mycoplasma* with descriptions of '*Candidatus* *Mycoplasma haemofelis*', '*Candidatus*  
472 *Mycoplasma haemomuris*', '*Candidatus* *Mycoplasma haemosuis*' and '*Candidatus*  
473 *Mycoplasma wenyonii*'. *Int J Syst Evol Microbiol* 51, 891-899.
- 474 Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 2002, Revision of  
475 haemotrophic *Mycoplasma* species names. *Int J Syst Evol Microbiol* 52, 683.
- 476 Nonaka, N., Thacker, B.J., Schillhorn van Veen, T.W., Bull, R.W., 1996, *In vitro*  
477 maintenance of *Eperythrozoon suis*. *Vet Parasitol* 61, 181-199.
- 478 Peters, I.R., Helps, C.R., Willi, B., Hofmann-Lehmann, R., Tasker, S., 2008, The  
479 prevalence of three species of feline haemoplasmas in samples submitted to a  
480 diagnostics service as determined by three novel real-time duplex PCR assays. *Vet*  
481 *Microbiol* 126, 142-150.
- 482 Rosse, W.F., Adams, J.P., 1980, The variability of hemolysis in the cold agglutinin  
483 syndrome. *Blood* 56, 409-416.
- 484 Smith, J.E., Cipriano, J.E., Hall, S.M., 1990, In vitro and in vivo glucose consumption  
485 in swine eperythrozoonosis. *Zentralblatt fur Veterinarmedizin - Reihe B* 37, 587-592.

- 486 Sutton, R.H., 1976, *Eperythrozoon ovis*: the difference in carbohydrate metabolism  
487 between infected and uninfected sheep erythrocytes. *Aust J Exp Biol Med Sci* 54,  
488 449-458.
- 489 Sutton, R.H., 1977, The effect of *Eperythrozoon ovis* infection on the glucose level  
490 and some acid-base factors in the venous blood of sheep. *Aust Vet J* 53, 478-481.
- 491 Sykes, J.E., Terry, J.C., Lindsay, L.L., Owens, S.D., 2008, Prevalences of various  
492 hemoplasma species among cats in the United States with possible hemoplasmosis. *J*  
493 *Am Vet Med Assoc* 232, 372-379.
- 494 Tasker, S., Caney, S.M.A., Day, M.J., Dean, R.S., Helps, C.R., Knowles, T.G., Lait,  
495 P.J.P., Pinches, M.D.G., Gruffydd-Jones, T.J., 2006a, Effect of chronic FIV infection,  
496 and efficacy of marbofloxacin treatment, on '*Candidatus Mycoplasma*  
497 *haemominutum*' infection. *Microbes Infect* 8, 653-661.
- 498 Tasker, S., Caney, S.M.A., Day, M.J., Dean, R.S., Helps, C.R., Knowles, T.G., Lait,  
499 P.J.P., Pinches, M.D.G., Gruffydd-Jones, T.J., 2006b, Effect of chronic FIV infection,  
500 and efficacy of marbofloxacin treatment, on *Mycoplasma haemofelis* infection. *Vet*  
501 *Microbiol* 117, 169-179.
- 502 Tasker, S., Helps, C.R., Day, M.J., Gruffydd-Jones, T.J., Harbour, D.A., 2003, Use of  
503 Real-Time PCR to detect and quantify *Mycoplasma haemofelis* and '*Candidatus*  
504 *Mycoplasma haemominutum*' DNA. *J Clin Microbiol* 41, 439-441.
- 505 Tasker, S., Peters, I.R., Day, M.J., Willi, B., Hofmann-Lehmann, R., Gruffydd-Jones,  
506 T.J., Helps, C.R., 2009, Distribution of feline haemoplasmas in blood and tissue  
507 following experimental infection. In preparation.
- 508 Westfall, D.S., Jensen, W.A., Reagan, W.J., Radecki, S.V., Lappin, M.R., 2001,  
509 Inoculation of two genotypes of *Haemobartonella felis* (California and Ohio variants)

510 to induce infection in cats and the response to treatment with azithromycin. *Am J Vet*  
511 *Res* 62, 687-691.

512 Willi, B., Boretti, F.S., Baumgartner, C., Tasker, S., Wenger, B., Cattori, V., Meli,  
513 M.L., Reusch, C.E., Lutz, H., Hofmann-Lehmann, R., 2006, Prevalence, risk factor  
514 analysis, and follow-up of infections caused by three feline hemoplasma species in  
515 cats in Switzerland. *J Clin Microbiol* 44, 961-969.

516 Willi, B., Boretti, F.S., Cattori, V., Tasker, S., Meli, M.L., Reusch, C., Lutz, H.,  
517 Hofmann-Lehmann, R., 2005, Identification, molecular characterisation and  
518 experimental transmission of a new hemoplasma isolate from a cat with hemolytic  
519 anaemia in Switzerland. *J Clin Microbiol* 43, 2581-2585.

520 Zachary, J.F., Smith, A.R., 1985, Experimental porcine eperythrozoonosis: T-  
521 lymphocyte suppression and misdirected immune responses. *Am J Vet Res* 46, 821-  
522 830.

523 Zulty, J.C., Kociba, G.J., 1990, Cold agglutinins in cats with haemobartonellosis. *J*  
524 *Am Vet Med Assoc* 196, 907-910.

525



526 **Table 1: Coombs' test results for cats in Group HF.**

527 0 indicates negative. Numbers indicate the inverse of a positive titre to that reagent in the  
528 Coombs' test. Agg indicates agglutination present in all wells. Results for Coombs' testing  
529 done on Days 71, 78 and 85 PI are not shown as all cats were negative in all wells.  
530 PZ indicates prozone effect; this is observed when the concentration of antibody in the well  
531 is too high (i.e. at lower dilutions of Coombs' antisera) to allow agglutination to occur. If  
532 the sample is diluted, agglutination then occurs. The lack of agglutination at high  
533 concentrations of antibodies is called the prozone effect. Lack of agglutination in the  
534 prozone is due to antibody excess resulting in very small complexes that do not clump to  
535 form visible agglutination.

536

537

538 **Figure captions**

539 **Figure 1**

540 Haemoplasma copy numbers recorded during the study against time DPI. Copy numbers  
541 shown per ml of blood.

542 **A:** *M. haemofelis* copy number/ml of blood for each cat in Group HF plotted against time  
543 DPI, with mean of Group HF plotted as continuous line.

544 **B:** '*Candidatus M. haemominutum*' and '*Candidatus M. turicensis*' copy number/ml of  
545 blood for each cat in Groups HM and TU plotted against time DPI, with mean of Groups  
546 HM and TU plotted as continuous lines.

547 Group TU had significantly lower copy numbers than both Groups HF ( $P<0.001$ ) and  
548 HM ( $P<0.001$ ).

549 **Figure 2**

550 Selected haematological variables during the study against time DPI; horizontal lines  
551 represent upper and lower reference range limits for each variable.

552 **A:** PCV values for each cat in Group HF plotted against time DPI, with mean of Group  
553 HF plotted as continuous line. Reference range: 25-45%.

554 **B:** PCV values for each cat in Groups HM and TU plotted against time DPI, with mean  
555 of Groups HM and TU plotted as continuous lines. Reference range: 25-45%.

556 Group HF had significantly lower PCVs than both Groups HM ( $P=0.018$ ) and TU  
557 ( $P=0.001$ ).

558 **C:** Haemoglobin values for each cat in Group HF plotted against time DPI, with mean of  
559 Group HF plotted as continuous line. Reference range: 8-15 g/dl.

560 **D:** Haemoglobin values for each cat in Groups HM and TU plotted against time DPI,  
561 with mean of Groups HM and TU plotted as continuous lines. Reference range: 8-15  
562 g/dl.

563 Group HF had significantly lower haemoglobin values than both Groups HM (P=0.005)  
564 and TU (P<0.001).

565 **E:** Mean cell volumes for each cat in Group HF plotted against time DPI, with mean of  
566 Group HF plotted as continuous line. Reference range: 40-55 fl.

567 **F:** Mean cell volumes for each cat in Groups HM and TU plotted against time DPI, with  
568 mean of Groups HM and TU plotted as continuous lines. Reference range: 40-55 fl.

569 Group HF had significantly higher MCVs than both Group HM (P<0.001) and TU  
570 (P<0.001).

571 **G:** White blood cell counts for each cat in Group HF plotted against time DPI, with mean  
572 of Group HF plotted as continuous line. Reference range: 4.9-19 x 10<sup>9</sup>/l.

573 **H:** White blood cell counts for each cat in Groups HM and TU plotted against time DPI,  
574 with mean of Groups HM and TU plotted as continuous lines. Reference range: 4.9-19 x  
575 10<sup>9</sup>/l.

576 White blood cell counts were not significantly different between the groups (P=0.306).

577 **Figure 3**

578 Graphs showing the collated autoagglutination and Coombs' test data for all 10 Group  
579 HF cats, together with the Group HF mean haemoglobin values (reference range 8-15  
580 g/dl) for temporal comparison to the time of onset of anaemia. Note that if persistent  
581 autoagglutination was present, as shown by autoagglutination in the PBS control wells,  
582 the class of the antibody involved, at the temperature in which autoagglutination was  
583 demonstrated, could not be recorded. Hence cats with positive tests at either 4°C or 37°C  
584 will be graphically represented in either the autoagglutination bar chart or the IgG/IgM  
585 bar charts, but not both.

586 **A:** Number of cats in Group HF showing persistent autoagglutination, positive IgM titres  
587 or positive IgG titres at 4°C alongside Group HF mean haemoglobin concentration.

588 **B:** Number of cats in Group HF showing persistent autoagglutination, positive IgM titres  
589 or positive IgG titres at 37°C alongside Group HF mean haemoglobin concentration.

590 **Figure 4**

591 Blood glucose measurements during the study against time DPI; horizontal lines  
592 represent upper and lower reference range limits. Reference range: 3-5.5 mmol/l.

593 **A:** Blood glucose measurements for each cat in Group HF plotted against time DPI, with  
594 mean of Group HF plotted as continuous line.

595 **B:** Blood glucose measurements for each cat in Groups HM and TU plotted against time  
596 DPI, with mean of Groups HM and TU plotted as continuous lines.

597 Group TU had significantly lower blood glucose concentrations than both Groups HF  
598 (P=0.006) and HM (P=0.027).

Figure 1

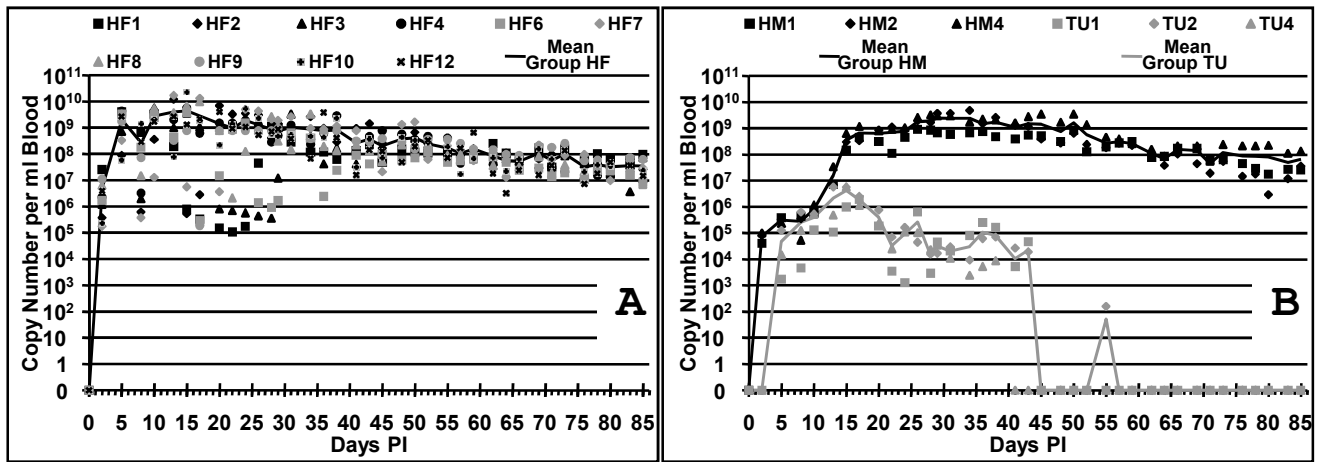


Figure 2

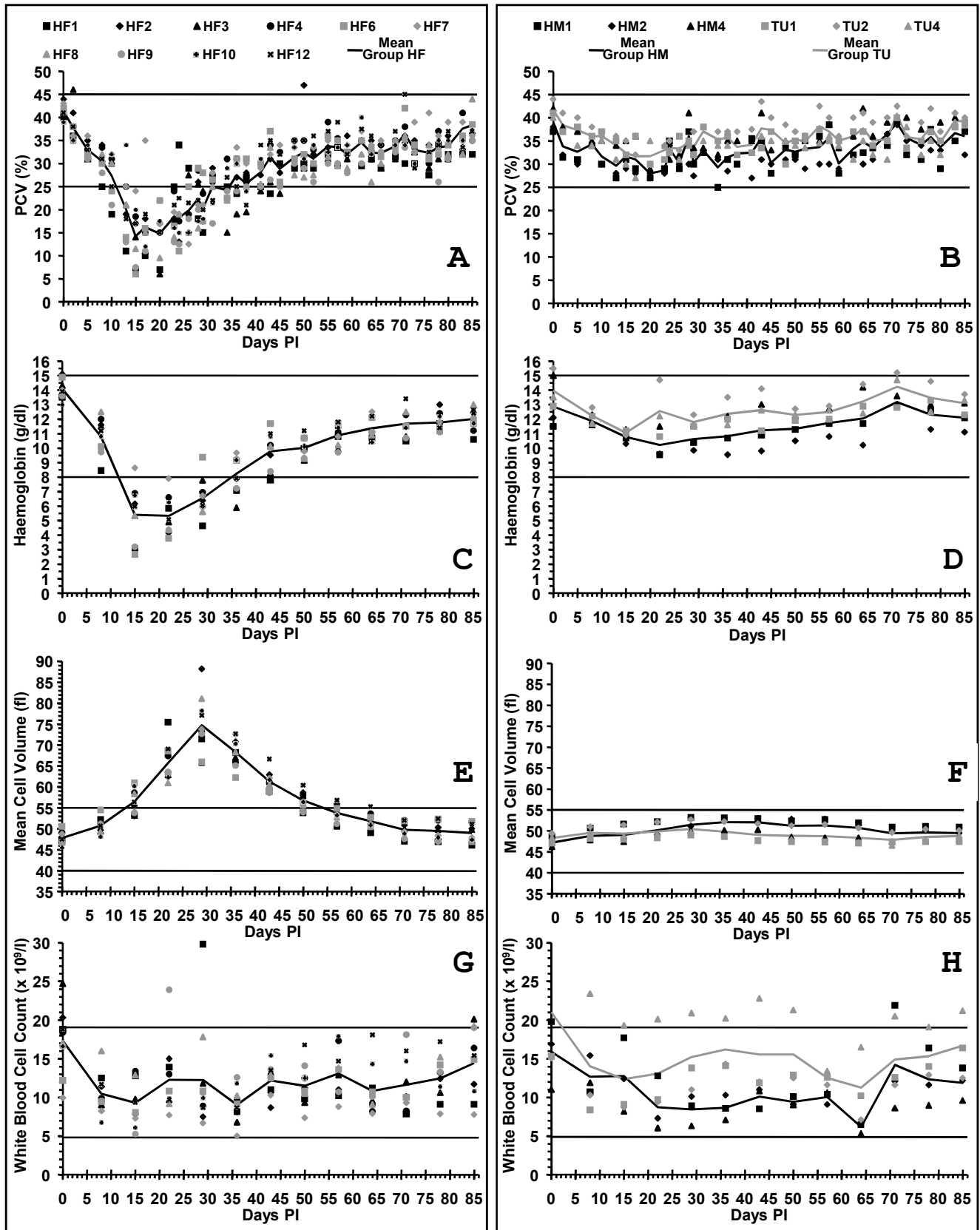


Figure 3

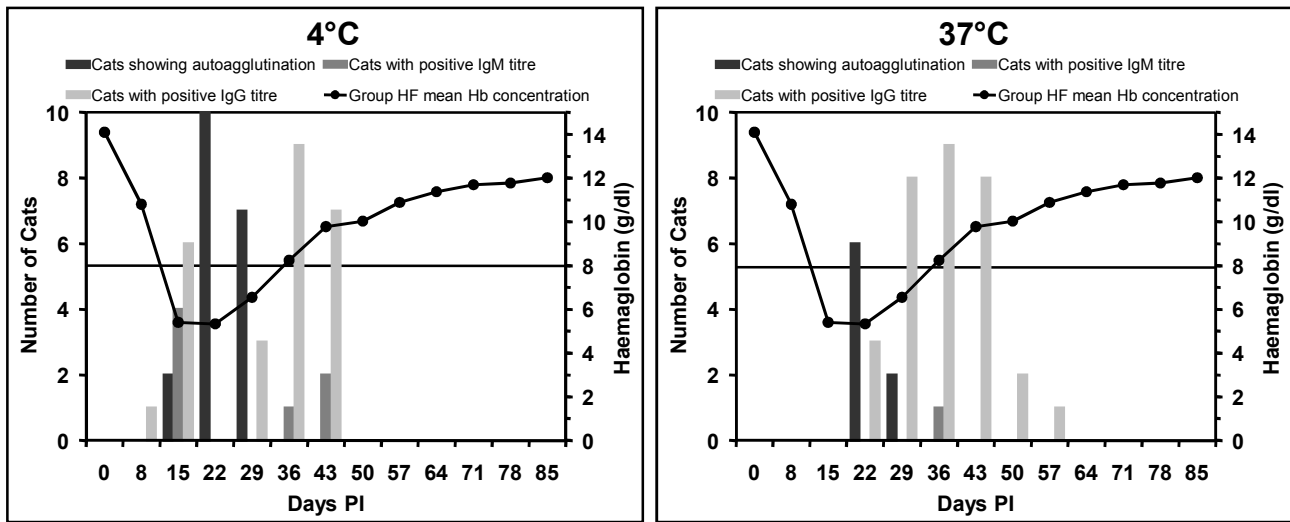
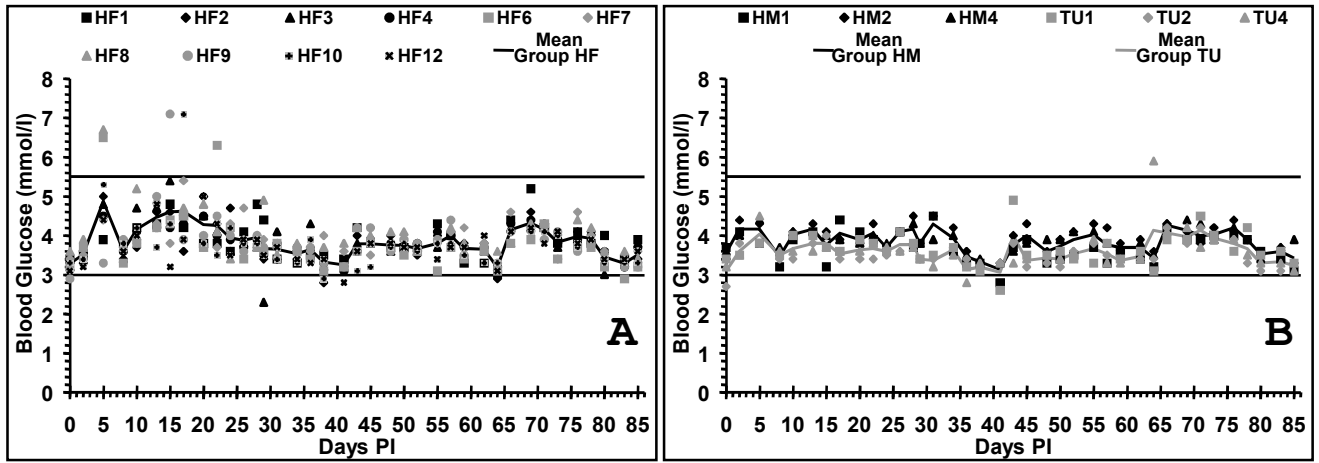


Figure 4





Cat ID	Day PI	0		8		15		22		29		36		43		50		57		64			
		Coombs' test	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	
HF1	Polyclonal	0	0	160	0	Agg	0	Agg	20	Agg	80	0	0	0	0	0	0	0	0	0	0	0	
	IgM	0	0	0	0	Agg	0	Agg	0	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	80	0	Agg	0	Agg	160	Agg	320	160	80	0	0	0	0	0	0	0	0	0	
	PBS	0	0	0	0	Agg	0	Agg	0	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
HF2	Polyclonal	0	0	0	0	80	0	Agg	Agg	Agg	Agg	20	20	0	20	0	0	0	0	0	0	0	
	IgM	0	0	0	0	≥640	0	Agg	Agg	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	320	0	Agg	Agg	Agg	Agg	320	160	160	80	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	Agg	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	
HF3	Polyclonal	0	0	0	0	40	0	Agg	Agg	160	40	40	20	20	20	20	0	20	0	0	0	0	
	IgM	0	0	0	0	320	0	Agg	Agg	0	0	≥640	320	20	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	160	0	Agg	Agg	≥640	≥640	320	160	320	160	320	160	0	≥640	0	160	0	0
	PBS	0	0	0	0	0	0	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	0	
HF4	Polyclonal	0	0	0	0	40	0	Agg	Agg	80	40	20	20	0	0	0	0	0	0	0	0	0	
	IgM	0	0	0	0	320	0	Agg	Agg	0	0	0	0	20	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	80	0	Agg	Agg	≥640	320	320	160	320	320	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	0	
HF6	Polyclonal	0	0	0	0	Agg	0	Agg	Agg	320	20	0	0	0	20	0	0	0	0	0	0	0	
	IgM	0	0	0	0	Agg	0	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	Agg	0	Agg	Agg	≥640	160	160	80	≥640	320	0	0	0	0	0	0	0	
	PBS	0	0	0	0	Agg	0	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	0	
HF7	Polyclonal	0	0	0	0	0	0	Agg	0	Agg	40	20	20	40	40	0	0	0	0	0	0	0	
	IgM	0	0	0	0	0	0	Agg	0	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	0	0	Agg	0	Agg	≥640	≥640	≥640	≥640	≥640	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	0	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
HF8	Polyclonal	0	0	0	0	20	0	Agg	Agg	Agg	80	0	20	40	40	0	20	0	0	0	0	0	
	IgM	0	0	0	0	0	0	Agg	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	320	0	Agg	Agg	Agg	≥640	≥640	≥640	≥640	≥640	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
HF9	Polyclonal	0	0	0	0	80	0	Agg	0	Agg	20	0	0	0	0	0	0	0	0	0	0	0	
	IgM	0	0	0	0	≥640	0	Agg	0	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	80	0	Agg	80	Agg	320	0	0	0	0	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	0	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
HF10	Polyclonal	0	0	0	0	0	0	Agg	30	Agg	Agg	20	0	0	0	0	0	0	0	0	0	0	
	IgM	0	0	0	0	0	0	Agg	0	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	0	0	Agg	320	Agg	Agg	160	160	160	160	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	0	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	
HF12	Polyclonal	0	0	0	0	20	0	Agg	Agg	Agg	80	0	0	0	20	0	0	0	0	0	0	0	
	IgM	0	0	0	0	0	0	Agg	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	
	IgG	0	0	0	0	80	0	Agg	Agg	Agg	≥640	≥640	320	0	≥640	0	0	0	0	0	0	0	
	PBS	0	0	0	0	0	0	Agg	Agg	Agg	0	0	0	0	0	0	0	0	0	0	0	0	