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1 **Insights into the dynamics of endemic *Coxiella burnetii* infection in cattle by application of**
2 **phase-specific ELISAs in an infected dairy herd**

3

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10

11 **Abstract**

12 Serological diagnosis of acute and chronic Q fever in humans relies on detection of antibodies to
13 phase I (PhI) and II (PhII) antigens of *Coxiella (C.) burnetii*. Although phase-specific antigens
14 are available, they are not yet used in ruminants as they are in humans. This study focuses on
15 phase-specific serology as a tool for analysis of the dynamics of infection in cattle. As a
16 prerequisite, sero-prevalence in Bavarian cattle (1) and sero-prevalences for age-groups (2) were
17 determined by ELISA (CHEKIT Q-Fever; mix of PhI/PhII-antigen). Subsequently, phase-
18 specific antigens were coated onto ELISA plates individually and tests were simultaneously
19 applied in an endemically infected herd with about 90 dairy cows and 250 calves/heifers in April
20 2005, March 2006 and retrospectively in May and October 2004. From April 2005 onward,
21 placentas were analysed for *C. burnetii* by PCR (3).

22 (1) Sero- and herd prevalences based on 21,051 sera from 603 Bavarian dairy farms collected in
23 2003 were 14.8% +/-0.48% and 72.3% +/-3.6%, respectively. (2) Analysis of 3965 animals from
24 105 farms for which age was reported revealed a base level of sero-prevalence of less than 5% in
25 1-2 years old animals, it increased to 15% in 2-3 years old and reached a plateau (25-30%) in
26 cows four years and older. (3) In May 2004 and April 2005 a peak of PhI⁻/PhII⁺-prevalence in

27 primiparous cows (2.0-3.5 years) was observed; but not in October 2004 and March 2006. The
28 PhI⁻/PhII⁺-pattern in primiparous cows changed to negative (one-third), PhI⁺/PhII⁺ (1/3) or
29 persisted (1/3). In contrast, sero-conversion was rare in multiparous cows (>3.5 years). If the PhI⁻
30 /PhII⁺ pattern was detected, it was due to an infection in preceding years. This pattern persisted
31 (2/3) or changed to negative (1/3); a change to PhI⁺/PhII⁺ did not occur. PhI⁻/PhII⁺ in heifers (1-2
32 yrs) always changed to negative. Detection of PhII-antibodies was significantly associated with
33 PCR-positive placentas. Remarkably, 45% of sera with the PhI⁻/PhII⁺ pattern were negative for
34 the CHEKIT Q-Fever ELISA, thus this test missed an important group of infected animals.

35 **Key words:** Q fever, serology, cattle, phase I, phase II

36 **Introduction**

37 *C. burnetii* is a small gram-negative obligate intracellular bacterium. Sporulation preserves its
38 infectivity even under harsh conditions outside the host (McCaul, 1991). Acute (e.g., pneumonia)
39 and severe chronic infections (e.g., endocarditis, granulomatous hepatitis) are of concern in
40 humans (reviewed by Fournier et al., 1998). In contrast, subclinical infection is common in
41 cattle. If infection occurs, it is generally related to reduced reproductive performance rather than
42 to abortion (To et al., 1998; Sting et al., 2000; Rodolakis et al., 2007), although an increased rate
43 of abortion in primiparous cows has been related to *C. burnetii* infection (Hässig and Lubsen,
44 1998). Sheep and goats are regarded as a major source of human infection with the organism
45 (Dupuis et al., 1987; Hellenbrand, 2001; Berri et al., 2002; Henning et al., 2009; Delsing and
46 Kullberg, 2008). In contrast, human infections are rarely attributed to cattle (Hellenbrand, 2001),
47 although cattle, once infected and sero-positive, are regarded as persistently infected for life
48 (Aitken, 1989; Lang, 1990).

49 Acute and chronic infections in humans have to be discriminated because of differences in
50 prognosis and therapy. This is achieved serologically by utilising a phenomenon displayed by *C.*
51 *burnetii* called phase variation: *C. burnetii* exists in two antigenic phases (PhI and PhII). Phase
52 variation is similar to the rough-smooth variation in enterobacteria and is due to a partial loss of

53 lipopolysaccharide (LPS) (Hackstadt et al., 1985; Toman et al., 1996). PhII-antibodies are
54 produced early after infection with virulent *C. burnetii*, whereas the increase of PhI-titre is
55 delayed (Dupuis et al., 1985; Williams et al., 1986). A low level of PhI-antibodies and a decrease
56 in levels of PhII-antibodies is a common finding months after acute infection. On the other hand,
57 cases of chronic endocarditis are characterised by low or absent reactivity of IgM and a strong
58 IgG- and IgA-response against both PhI and II antigens (Worswick and Marmion, 1985). The
59 immunofluorescence antibody test (IFAT) is regarded as a gold standard for serological
60 diagnosis of Q-fever in humans (Fournier et al., 1998). In contrast, the complement fixation test
61 (CFT) is still frequently used for serological diagnosis in ruminants (Sting et al., 2004);
62 furthermore, CFT is used as a standard for validation of ELISA, although its sensitivity is known
63 to be low (Kittelberger et al., 2009).

64 Because of the endemic nature, we sought to analyse the dynamics of infection within dairy
65 farms. Initially, sero-prevalence and age-related sero-prevalences were determined by a
66 commercial ELISA (CHEKIT Q-Fever; Idexx, Ludwigsburg). Subsequently, one endemically
67 infected dairy farm was analysed for phase-specific antibodies.

68 **Materials and Methods**

69 ELISA: Three types of ELISA were included in the present study: a commercially available
70 indirect ELISA using a mixture of PhI and -II-antigens (CHEKIT Q-Fever, Idexx, Ludwigsburg)
71 and two prototypes with PhI- and -II-antigens coated separately (PhI- and PhII-ELISA). PhI- and
72 PhII-coated plates were provided by Idexx: antigens, their concentrations and coating conditions
73 were the same as for CHEKIT Q-Fever. The same negative and positive controls were used. The
74 test was performed according to CHEKIT Q-Fever instructions. OD% between 30-40% was
75 scored as equivocal; OD% values less than 30% and higher than 40% were interpreted as
76 negative and positive, respectively. The cut-off for the phase-specific ELISA was 40%. The
77 monoclonal peroxidase conjugate recognises bovine IgG1.

78 PCR: DNA from placentas was purified using the QIAmp[®] Mini-Kit (Qiagen, Hilden).
79 Polymerase Chain Reaction (PCR) was performed according to Willems et al. (1994).
80 Handling of blood samples: Blood samples included in this study were centrifuged (2000 rpm,
81 20 minutes); the serum was collected and frozen at -20°C until testing.
82 Assessment of seroprevalence: To determine sero-prevalence and herd prevalence of antibodies
83 directed against *C. burnetii* in Bavaria, 21,051 sera from 603 herds were tested using the
84 CHEKIT Q-Fever ELISA. Selection of herds was proportional to the number of cattle per
85 district. Only herds with at least 10 animals were included; all animals per herd for which
86 samples were available were tested. The age of the animals used was >18 months.
87 Assessment of sero-prevalence for age-groups: For analysis of age-related effects of sero-
88 prevalence, 3,965 sera from 105 farms were analysed with the CHEKIT Q-Fever ELISA.
89 Prevalence was plotted over age-groups.
90 Herd A, an endemically infected dairy farm: Herd A is a dairy farm with about 90 cows and 250
91 calves/heifers. Cows are kept in two groups: a freestall barn and a smaller tie-stall. Calving
92 occurs in six boxes. Calves are removed from the cow after birth; they are kept single for the first
93 days and are housed in groups in a barn separated from that with cows. Cattle older than four
94 months are reared on a second location about 50 km away. They return the first time for breeding
95 and again 6-8 weeks before calving. During these periods of return animals are kept away from
96 cows in a separate barn; two weeks before calving pregnant heifers are introduced into the cow
97 herd for acclimatisation.
98 *C. burnetii* had been detected sporadically over the past years in herd A. A case of abortion
99 occurred (5.4.2005) and *C. burnetii* was detected by PCR in the aborted foetus. The farmer
100 agreed to join the present study in April 2005.
101 Placental material (n = 67) was collected from a proportion of cows between April 2005 and
102 March 2006 in order to determine the rate of cows shedding *C. burnetii* at birth.

103 Blood samples were collected in April 2005 and March 2006: 28.4.2005 (n=344) and 1.3.2006
104 (n=268). Sera from May (18.5.2004; n=92) and October 2004 (21.10.2004; n=88) had been
105 stored at -20°C and were analysed retrospectively. These sera had been collected within a
106 different project; therefore young animals were not included.

107 Sera were analysed by phase specific ELISAs. Samples were interpreted by phase patterns: PhI⁻
108 /PhII⁻, PhI⁻/PhII⁺, PhI⁺/PhII⁻ and PhI⁺/PhII⁺.

109 Based on all animals for which age had been reported the prevalences (%) of the phase-patterns
110 per age-group were calculated (respective phase pattern positive/analysed animals per group).

111 Those animals which had been tested on two subsequent points in time (i.e. May 2004 versus
112 (vs) October 2004, October 2004 vs April 2005 and April 2005 vs March 2006) were selected
113 and prevalence (%) of phase-pattern (PhI⁻/PhII⁺ and PhI⁺/PhII⁺) was determined and plotted over
114 age-groups at the first respective point in time of sampling.

115 For individual animals the change of phase pattern was determined for the following groups:
116 heifers (1-2 yrs), primiparous cows (2-3.5 yrs) and multiparous cows (>3.5 yrs). For 139 cows
117 data of at least two subsequent samplings were available, whereby 62, 12 and 65 cows had been
118 tested four, three and two times, respectively. 275 comparisons on 139 animals have been
119 performed. The age at the first respective sampling was used for classification of age groups.

120 PCR- and phase pattern results were compared. For 32 cows of 67, which had been analysed by
121 PCR, results on phase patterns in April 2005 and March 2006 were available.

122 Finally, phase patterns were compared to CHEKIT Q fever. Therefore results of 517 sera which
123 had been tested with phase-specific ELISAs and CHEKIT Q fever were compared.

124 Statistical analysis of the data was performed with MedCalc[®] version 9.5.2.0. (MedCalc
125 Software, Broekestraat 52, B-9030 Mariakerke, Belgium).

126 **Results**

127 Because data on antibody prevalence for Bavaria were incomplete and relied on CFT, sero- and
128 herd prevalences were assessed as a prerequisite for any further investigations on the dynamics

129 of infection. Sero- and herd prevalences were 14.8% (CI95%: 14.3-15.3%) and 72.3% (CI95%:
130 68.7-75.9%), respectively. Fig. 1 shows the relative distribution of herds according to intra-herd-
131 prevalence (IHP%).

132 Analysis of 3,965 animals with the CHEKIT Q-Fever ELISA revealed age-related effects. In Fig.
133 2, rates of positive sera are plotted over age groups. A baseline level below 5% was observed in
134 animals 1 to 2 yrs old, followed by a sharp increase in the next age group, while further increases
135 were less pronounced.

136 In April 2005, material from an aborted foetus from herd A was submitted for analysis. Placental
137 material was positive for *C. burnetii* by PCR. The number of analysed placentas and the
138 cumulative number of positive samples, as determined by PCR, are summarised in Fig. 3. The
139 steepest increase was reported for July and August 2005, while no further increase was observed
140 between October 2005 and March 2006. Except for the initial case the detection of *C. burnetii* in
141 placental tissue was not associated with abortion.

142 Because an age-related sero-conversion was observed (Fig. 2), serological profiles of three
143 possible phase-patterns ($\text{PhI}^-/\text{PhII}^+$, $\text{PhI}^+/\text{PhII}^+$, $\text{PhI}^+/\text{PhII}^-$) were plotted over age groups (Fig. 4a-
144 d). A striking regularity of phase-patterns was visible: a peak in the $\text{PhI}^-/\text{PhII}^+$ -pattern was
145 observed in cows 2.6-3.0 yrs old in May 2004 and April 2005, but this peak was absent in
146 October 2004 and almost absent in March 2006. A second peak of this phase-pattern was
147 observed in cows 3.6 to 4.0 yrs old on both occasions in 2004. This second peak was present in
148 April 2005, as a shoulder of the first peak. A characteristic peak of the $\text{PhI}^+/\text{PhII}^+$ -pattern in
149 animals 3.1-3.5 yrs old was regularly observed. Its dominance in March 2006 has to be stressed.
150 Finally, the $\text{PhI}^+/\text{PhII}^-$ -pattern was rarely detected.

151 Calves and young cattle were only analysed in April 2005 and March 2006, respectively. The
152 $\text{PhI}^-/\text{PhII}^+$ -reactivity in cattle 1.0-2.0 yrs old was only observed in April 2005 and was
153 completely absent in the following year. In contrast to April 2005 (Fig. 4c), the $\text{PhI}^-/\text{PhII}^+$ -

154 pattern was absent in cattle between 1.0 and 2.0 years of age, and the $\text{PhI}^+/\text{PhII}^+$ pattern was
155 significantly reduced in animals 2.1-2.5 yrs old.

156 For better comparison of changes in phase-pattern between two samplings, further analysis
157 focused on those animals that were analysed at two subsequent points in time. The age at the
158 respective first sampling was used as the variable for presentation of prevalence (Fig. 5a-c). An
159 age-related regularity became obvious: in the 2.0-3.0 yrs age group, the prevalence of the PhI^-
160 $/\text{PhII}^+$ pattern decreased and that of the $\text{PhI}^+/\text{PhII}^+$ pattern increased (Fig. 5a and c). Notably, this
161 finding was not observed between October 2004 and April 2005 (Fig. 5b). The situation in cows
162 3.0-4.0 yrs old is best described as a nodal point, because prevalences were close together and
163 they did not change. In animals exceeding the age of 4 yrs, a clear divergence of both phase-
164 patterns was characteristic, with minimal change of prevalence between both points in time.
165 Surprisingly, a 10% decrease of $\text{PhI}^+/\text{PhII}^+$ -prevalence was observed between October 2004 and
166 April 2005 (Fig. 5b).

167 Results of individual animals from two age-groups (2.0-3.5 years; >3.5 years) at two subsequent
168 points in time were compared (Table 1). Analysis of animals with the $\text{PhI}^-/\text{PhII}^+$ pattern at the
169 first point in time revealed three things: First, cattle 1.0-2.0 yrs old always lost their PhII^- -
170 reactivity and became negative (not included in Table 1). Second, for cattle 2.0-3.5 yrs old, three
171 outcomes with equal likelihood were possible: one-third switched to $\text{PhI}^+/\text{PhII}^+$, one-third
172 became negative and the last third remained as $\text{PhI}^-/\text{PhII}^+$. Finally, cows older than 3.5 yrs at the
173 time of the first test became negative (one-third) or remained $\text{PhI}^-/\text{PhII}^+$ (two-thirds), but a
174 transition to $\text{PhI}^+/\text{PhII}^+$ was never observed. A transition from negative to $\text{PhI}^-/\text{PhII}^+$ ($n = 5$) or
175 $\text{PhI}^+/\text{PhII}^+$ ($n = 10$) was observed in young cows (<3.5 yrs), whereas it was rare in old cows
176 (>3.5 yrs). Although the $\text{PhI}^+/\text{PhII}^-$ pattern was a rare finding, seven multiparous cows with the
177 $\text{PhI}^+/\text{PhII}^-$ pattern subsequently became negative. For 10 animals that were $\text{PhI}^+/\text{PhII}^-$ at the
178 second point in time, this pattern developed from $\text{PhI}^+/\text{PhII}^+$ (6) or was due to persistence (2).
179 Analysis of all animals with $\text{PhI}^+/\text{PhII}^-$ pattern revealed a median for $\text{PhI-OD}\%$ of 75%.

180 Persistence of the PhI/PhII⁺-pattern was observed in March 2006 in 9 cows that were older than
181 3.5 yrs in April 2005. Samples from May and October 2004 were available and analysed for 7
182 and 6 cows, respectively (Fig. 6; N° 1-7): two cows (N° 1 and 5) had PhI-antibodies in May
183 2004 but OD% decreased to levels below the cut-off. Another three cows (N° 3, 6 and 7) had
184 borderline PhI-antibodies: OD% decreased in cows N° 3 and 7, while for cow N° 6 an increase
185 in OD% was observed for both phases between October 2004 and April 2005.

186 Throughout this paper, phase-patterns were used for classification of sera. Samples from 164
187 cattle collected in April 2005 were compared with respect to PhII and PhI-OD%. Reactivity
188 between 30% and 40% was regarded as equivocal, whilst, in this study, sera with OD%≥40%
189 were scored as positive (Fig. 7). It becomes evident that for a few animals, there is only a gradual
190 difference between PhI⁻/PhII⁺ and PhI⁺/PhII⁺.

191 Sixty-seven placentas had been submitted for PCR. For 32 cows serological data from April
192 2005 and March 2006 were available. Phase-patterns in April 2005 and March 2006 were
193 compared and PCR-positive animals are shown in Table 2. *C. burnetii* was detected in 11 cases:
194 Four animals were 3.0-3.5, four 3.6-4.0 and three 4.9 to 5.3 years old in May 2005. *C. burnetii*
195 could be detected by PCR in 9 of 17 placentas of PhII⁺ cows in March 2006 but in only 6 of 13
196 PhI⁺ cows. Analysis of these data with Fisher's exact test revealed that PhII-antibodies were
197 significantly associated with detection of *C. burnetii* by PCR (p=0.027), whereas detection of
198 PhI-antibodies was not (p=0.243). In two sero-negative cows, *C. burnetii* was detected by PCR.
199 For these animals, data from 2004 were available as well. These data were negative at each
200 testing (Fig. 6; N° 7 and 8).

201 CHEKIT Q-Fever ELISA was compared with phase-patterns (Table 3). Overall, 52 (45%) of 115
202 samples with the PhI/PhII⁺ pattern were scored as negative with this test, when a cut-off of 40%
203 was used. Seventeen samples showed reactivity between 30-40%. Median, first and third quartile
204 for PhI (PhII) reactivity were 7.3 (79.5), 3.0 (58.0) and 14.0 (109.5), respectively. Samples with
205 PhI-reactivity were always scored as positive. Finally, eleven (2.9%) of 381 animals with PhI⁻

206 /PhII⁻ pattern scored positive in CHEKIT Q-Fever ELISA. For eight samples CHEKIT Q-Fever
207 OD% was between 40 and 50%, for the remaining three it was 69%, 97% and 111%,
208 respectively.

209 **Discussion**

210 To our knowledge, this is the first report on the use of phase-specific ELISA for the detection of
211 antibodies to *C. burnetii* in cattle. Because ELISA plates that came with the commercialised
212 CHEKIT Q-Fever were coated with a mix of PhI- and PhII-antigens, the separate coating of
213 these antigens was the easiest approach with which to assess the value of phase-specific
214 serology. PhI- and -II-ELISAs were prototype tests and a proper validation could not be
215 performed under field conditions. However, the same concentration of antigen as used for
216 CHEKIT Q-Fever ELISA was coated to test plates; furthermore, the same test protocol, controls
217 and cut-off values were used in this study.

218 Comparison of CHEKIT Q-Fever with prototype phase-specific ELISAs revealed that almost
219 50% of PhI/PhII⁺ sera tested negative in the former (Table 3). Apparently, CHEKIT Q-Fever
220 ELISA was inefficient in detecting PhII-antibodies. This is important since PhII-reactivity - not
221 PhI - was significantly associated with detection of *C. burnetii* by PCR (Table 2). PhII-antigens
222 are essential for serological diagnosis of Q fever. CHEKIT Q-Fever was criticised in the past for
223 its low sensitivity because *C. burnetii* was detected by PCR in sero-negative ruminants; this was
224 especially true for small ruminants but less obvious in cattle (Arricau-Bouvery and Rodolakis,
225 2005; Rodolakis et al., 2007; Kittelberger et al., 2009). A lack of sensitivity was attributed to the
226 use of the tick-derived Nine Mile strain of *C. burnetii* and, consequently, the use of a ruminant
227 strain in ELISA was recommended (Rodolakis et al., 2007). However, Guatteo et al. (2000)
228 implemented an ELISA (Cox Ruminants[®], LSI, Lisseux, France) based on a ruminant strain, and
229 they reported that this ELISA failed to detect antibodies in cows that were positive for *C.*
230 *burnetii* by PCR. Alternatively, the problem could be explained by a lack of PhII-antigens on
231 ELISA plates. Inefficient binding of the truncated PhII-LPS as compared to the PhI-antigen to

232 ELISA plates and/or competition of both antigens for adsorption might be critical in the context
233 of production (Hackstadt et al., 1985; Toman et al., 1996). Notably, an improved performance of
234 CHEKIT Q-Fever had been demonstrated recently (Kittelberger et al., 2009). Moreover, it has to
235 be kept in mind that ELISA was calibrated by CFT and on the other side the value of PCR-
236 positive results must be analysed critically. Last but not least, the immune response towards an
237 intracellular parasite - as discussed later - has to be considered, when differences between ELISA
238 and PCR are discussed.

239 Following infection in humans and guinea pigs the PhII-IgG response is detected before the
240 increase of PhI-IgG (Dupuis et al., 1985; Williams et al., 1986; Worswick and Marmion, 1985).

241 The absolute dominance of the PhI⁻/PhII⁺-pattern over PhI⁺/PhII⁺, as observed in April 2005
242 (Fig. 4c) in cows 2-3 yrs old, as well as a change in phase-pattern prevalence in this age-group
243 almost one year later (Fig. 4d, Fig. 5c), are in good agreement with early PhII- and late PhI-
244 antibodies. The probability of detecting *C. burnetii* should be higher in acutely infected cattle.
245 Indeed, a significant association between PhII-antibodies and detection of *C. burnetii* in placenta
246 by PCR was observed. Most remarkably, our data support the finding of Hässig and Lubsen,
247 (1998) that infection with *C. burnetii* was associated with abortion in primiparous but not in
248 multiparous cows. The initially observed abortion and an associated shedding of Coxiellae might
249 explain the dominance of PhI⁻/PhII⁺ in April 2005. But it doesn't explain the detection of PhI⁻
250 /PhII⁺-pattern in heifers which are kept at another location.

251 We assumed that we would observe a classical transition from sero-negative to PhI⁻/PhII⁺ (=
252 acute infection) to PhI⁺/PhII⁺ (= chronic infection). Surprisingly however, serological transition
253 of PhI⁻/PhII⁺ cattle was age-related: Animals that were one to two years old always became
254 negative upon subsequent testing. PhII-reactivity in these heifers might be explained by
255 maternally derived antibodies. We concluded that the PhI⁻/PhII⁺ pattern in heifers (10%) in 2005
256 reflected active infection with *C. burnetii* because PhII-reactivity was absent in the same age-
257 group in March 2006. Two of these animals tested negative by PCR at calving; although

258 additional heifers with the PhI⁻/PhII⁺ pattern need to be tested for shedding of *C. burnetii* at
259 parturition to confirm its absence, we assumed establishment of immunity in these animals. This
260 view is supported by vaccination trials in which protection against persistent infection could be
261 achieved by vaccination of non-pregnant animals before the first breeding (Woernle and Müller,
262 1986; Guatteo et al., 2008).

263 For primiparous cows (2.0-3.5 yrs), three possible outcomes were observed with equal
264 likelihood: Transition to negative (1/3), transition to PhI⁺/PhII⁺ (1/3) or persistence of the PhI⁻
265 /PhII⁺ pattern (1/3). Remarkably, detection of the PhI⁻/PhII⁺ pattern in multiparous cows (≥ 3.5
266 yrs) was due to persistence rather than to acute infection (Fig. 6). Only two possible outcomes
267 were observed: One-third still became negative, while two thirds persisted in this pattern. A
268 transition to PhI⁺/PhII⁺ was never observed.

269 A simple translation of PhI⁻/PhII⁺ and PhI⁺/PhII⁺ patterns of individual animals in terms like
270 'acute' and 'chronic', respectively, is inappropriate. However, the level of animals categorised as
271 PhI⁻/PhII⁺ seems to reflect the extent of 'acute infection' at the herd level. The probability of
272 detecting *C. burnetii* in PhII-positive animals at subsequent calving is 9/19 (47%; Tab. 2); it is
273 independent of the PhI⁻/PhII⁺ or PhI⁺/PhII⁺ pattern.

274 The important role of primiparous cows in endemically infected herds does not generally exclude
275 the possibility of infection and subsequent sero-conversion in older cows. Indeed, an increase of
276 sero-prevalence with age is demonstrated in Fig. 2. However, despite intensive shedding in the
277 summer 2005 and frequent seroconversion in primiparous cows about 60% of cows older than 4
278 yrs remained sero-negative (Fig. 5c and Table 1). Similar rates of sero-negative (ELISA) cows in
279 endemically infected herds were reported by Guatteo et al. (2000). We assumed that the majority
280 of these cows had already experienced infection and mounted protective immunity.
281 Environmental contamination (as a result of shedding in faeces, urine, vaginal mucus or by
282 placental material) with *C. burnetii* had already been demonstrated by Yanase et al. (1998). Thus
283 it is assumed that all animals within a barn were exposed. The assumption that at least some of

284 these multiparous cows could have built up an efficient cellular immunity with low or
285 undetectable levels of antibodies, as in mycobacterial infections, is supported by data obtained in
286 humans: Ascher et al. (1983) reported that only 14 (61%) of 36 skin-test positive people were
287 antibody positive by IFAT; a comparison with CFT revealed that only 52% of the people
288 positive upon skin-testing were also antibody-positive (Hustson et al., 2000). A transient PhI⁻
289 /PhII⁺-pattern, as observed for animals in this study, likely disappeared because *C. burnetii* was
290 successfully controlled.

291 What is the event determining the outcome of infection with *C. burnetii*? Pregnancy might be
292 important. During pregnancy, a shift of immunity towards T_H2-activity with elevated levels of
293 immunosuppressive IL-10 occurs. It is probably further enhanced by *C. burnetii* itself (Capo et
294 al., 1996). Such a polarisation of the immune response was described as the T_H1/T_H2-paradigm
295 in mice and humans (Wegmann et al., 1993; Romagnani, 1997). Not only does T_H2-activity
296 direct the immune response in an unfavourable direction to control an intracellular parasite, but
297 IL-10-mediated immunosuppression could also activate *C. burnetii* in persistently infected cows
298 during pregnancy. On the positive side, the benefit could be prevention of immune-mediated
299 placentitis and subsequent abortion. The hypothesis that pregnancy is an important trigger relies
300 on non-immune heifers at the time of first pregnancy.

301 About 50% of the foetuses in the endemically infected herd – PCR-positive results in PhI⁻/PhII⁺
302 and PhI⁺/PhII⁺ cows at calving (Table 2) - were considered to have been exposed to *C. burnetii*
303 during pregnancy. The amount of bacteria present in such cases exceeds that necessary for *post-*
304 *natal* infection by a multiple. What is the biological consequence of an *in-utero* infection? Sero-
305 prevalence decreased almost to zero in calves between 6-12 months (Fig. 2 and Fig. 4c), but a
306 transient antibody response due to *in-utero* infection would have been masked by maternally
307 derived antibodies. Moreover, such transient antibody responses were observed in older animals
308 (e.g., heifers). Additionally, a decrease of antibody titre in humans has to be regarded as a
309 common event; it is associated with a favourable prognosis. What is the impact of immunity after

310 foetal or *post- partum*-infection? From an evolutionary point of view, such immune animals
311 characterised by cellular immune response in the absence of detectable antibodies could be
312 crucial for maintenance of endemic infection in dairy farms with *C. burnetii*: These immune
313 animals would give birth to non-exposed, non-immune calves that could be infected as pregnant
314 primiparous cows, with subsequent persistence of infection. The steady-state between infected
315 sero-positive cows and immune sero-positive/–negative cows would sustain endemic infection at
316 the herd level. This would explain why within-herd prevalence rarely exceeded 50%, and why
317 prevalence that exceeded this value rather indicates active infection at the herd level (Fig. 1).
318 Any discussion of pregnancy as a possible trigger has to include the possibility that *in-utero*
319 infection and a shift towards T_H2 -immunity might favour the establishment of oral tolerance.
320 Finally, systemic immunotolerance as described for BVDV (Peterhans et al., 2003) has to be
321 considered when sero-negative and PCR-positive animals are discussed (Table 2; Fig. 6: N° 8
322 and 9).

323 The situation in one endemically infected dairy farm was described. Whether or not these
324 findings can be extrapolated remains to be determined. As a prerequisite of our work on phase-
325 specific serology, we analysed sero-prevalence in dairy cattle. Sero-prevalence was assessed in
326 earlier studies, but they were restricted to certain regions and relied on complement-fixing
327 antibodies: sero- and herd prevalences in the northern parts of Bavaria in 1986 were 7.6% and
328 30%, respectively (Roth and Bauer, 1986). Analysis of cattle sera south of the Bavarian Forest
329 revealed a prevalence of 12% (Rehacek et al., 1993). In the present study, an ELISA was
330 implemented: *C. burnetii*-specific antibodies were found in 14.8% of animals older than 18
331 months and in 72% of the tested herds. The higher prevalences observed in this study are most
332 likely explained by higher sensitivity of the ELISA (Kittelberger et al., 2009). Although infection
333 is widespread, IHP% was less than 10% in 64.1% of herds and exceeded 50% in only 6.1% of
334 the herds. The prevalence of sero-negative but immune cows, as hypothesised above, probably
335 limits that of persistently infected sero-positive animals, which might be important for steady-

336 state infection within herds. As a consequence, IHP% exceeding 70% is rarely observed (Fig. 1)
337 and might indicate recent herd infection.

338 This is the first report on phase-specific serology in cattle. The increase of sero-prevalence in
339 animals between two and three years (Fig. 2) was associated with an increased prevalence of PhI⁻
340 /PhII⁺-pattern. However, it has to be kept in mind that categorical classification as used in this
341 study simplified the data. In some cases only gradual differences were observed between certain
342 phase-patterns (Fig. 7). The authors are aware about critics that a validation of phase specific
343 ELISAs is still outstanding. However, validation relies on defined negative and positive sera; but
344 how to define these categories? Facing this uncertainty analysis of phase patterns for age-groups
345 was a rather practical approach. Currently, a representative number of dairy farms are under
346 investigation and a quantification of phase-specific antibodies, as in human medicine, will be
347 performed.

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- 438

1 Table 1: Transition of phase pattern: Animals in herd A were tested in May 2004, October 2004,
 2 April 2005 and March 2006. For 139 cows data of at least two subsequent samplings were
 3 available, whereby 62, 12 and 65 cows had been tested four, three and two times, respectively.
 4 275 comparisons on 139 animals have been performed. As an additional variable the age at the
 5 first respective sampling was included: Analysis was performed for primiparous cows (2.0-3.5
 6 yrs) and multiparous cows (>3.5 yrs; in brackets). The total of three comparisons (May04 vs
 7 Oct04, Oct04 vs Apr05 and Apr05 vs Mar06) is given whereby the first respective point in time
 8 of sampling is "1st Test" and the subsequent date is "2nd Test".

		2 nd Test				Σ
		PhI/PhII ⁻	PhI ⁺ /PhII ⁻	PhI/PhII ⁺	PhI ⁺ /PhII ⁺	
1 st Test	PhI/PhII ⁻	33 (60)	1 (0)	5 (1)	10 (1)	49 (62)
	PhI ⁺ /PhII ⁻	0 (5)	0 (2)	0 (0)	0 (0)	0 (7)
	PhI/PhII ⁺	10 (7)	1 (0)	11 (14)	1 (0)	32 (21)
	PhI ⁺ /PhII ⁺	2 (3)	3 (3)	4 (2)	31 (56)	40 (64)
	Σ	45 (75)	5 (5)	20 (17)	51 (57)	121 (154)

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1 Table 2: Comparison of phase-pattern and PCR-results: Between April 2005 and March 2006 67
 2 placentas were submitted for analysis of *C. burnetii*-specific subgenomic sequences by PCR. For
 3 32 cows phase patterns in April 2005 and March 2006 were available. The number of PCR-
 4 positive animals and the number of analysed placentas (in brackets) are recorded. Only those
 5 possibilities for which results were available are shown.

6

		2 nd Test (Mar 2006)				Σ
		PhI ⁻ /PhII ⁻	PhI ⁺ /PhII ⁻	PhI ⁻ /PhII ⁺	PhI ⁺ /PhII ⁺	
1 st Test (Apr 05)	PhI ⁻ /PhII ⁻	2(12)				2(12)
	PhI ⁺ /PhII ⁻	0(1)				0(1)
	PhI ⁻ /PhII ⁺	0(2)		2(3)	2(4)	4(9)
	PhI ⁺ /PhII ⁺			1(1)	4(9)	5(10)
	Σ	2(15)		3(4)	6(13)	11(32)

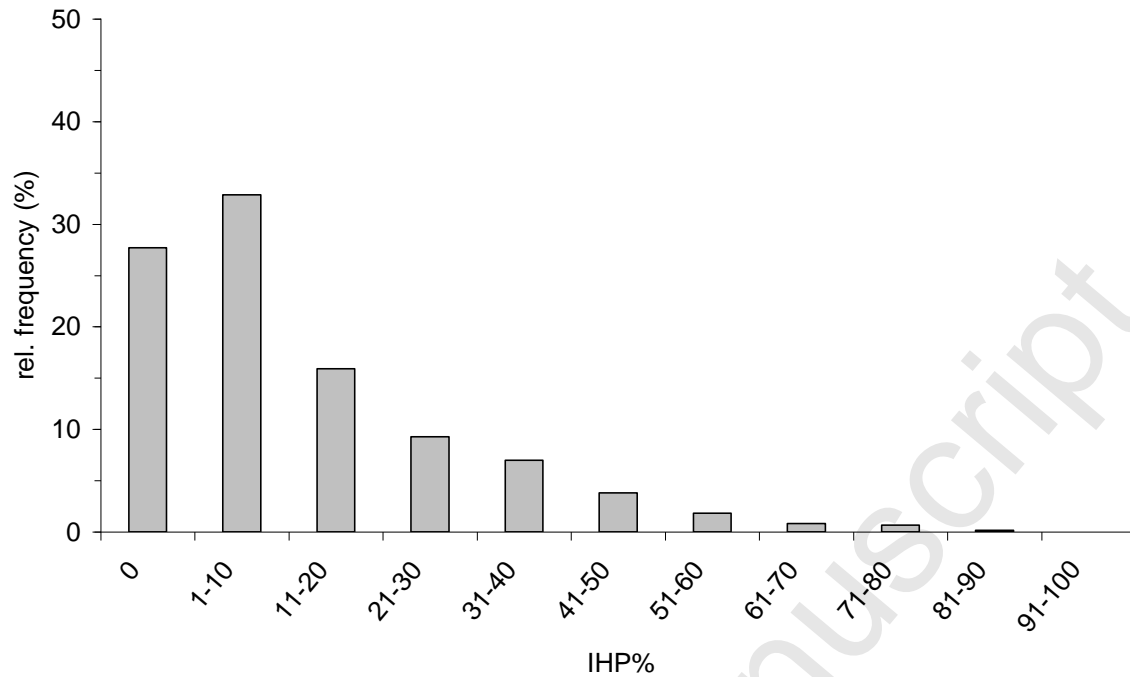
7

1 Table 3: Comparison of CHEKIT Q-Fever (CHEKIT QF) and phase-specific ELISAs: CHEKIT
2 QF test plates are coated with a mix of PhI and PhII antigens. For phase-specific testing antigens
3 were coated individually onto ELISA plates. Results in CHEKIT QF were compared with phase
4 patterns.

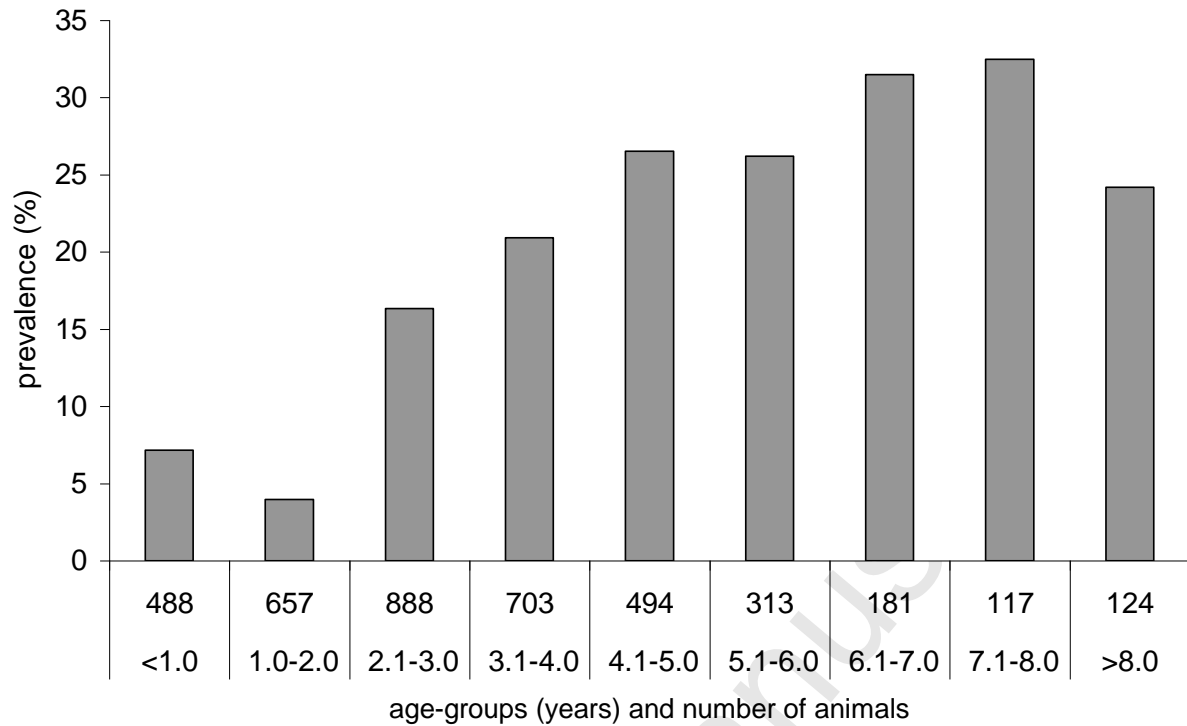
CHEKIT QF	PhI ⁻ /PhII ⁺	PhI ⁺ /PhII ⁺	PhI ⁺ /PhII ⁻	PhI ⁻ /PhII ⁻
neg	52	0	0	381
pos	63	57	6	11

5

6



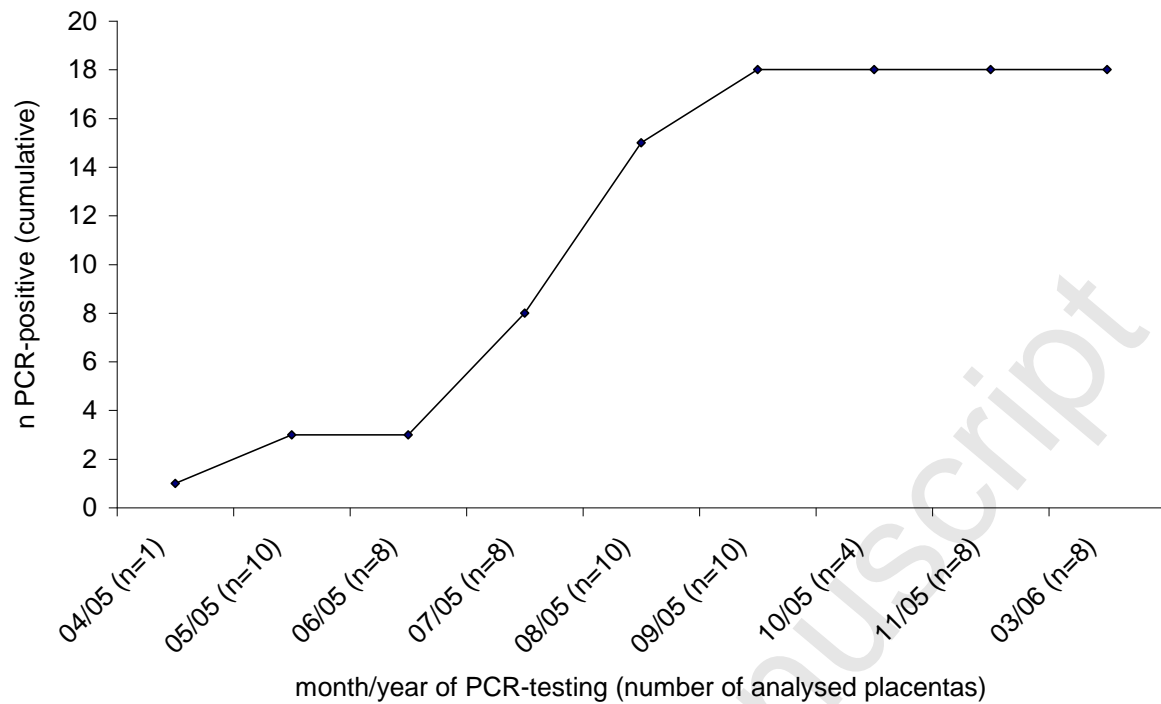
1
2 Figure 1: A total of 21,051 sera from 603 Bavarian dairy herds were analysed for antibodies to
3 *C. burnetii* (CHEKIT-Q-Fever ELISA; Idexx, Ludwigsburg). The minimum sample number per
4 herd was 10. For each herd, the relative intra-herd-prevalence (IHP%) of positive samples was
5 calculated. The distribution of herds over classes of IHP% is shown.



1
2 Figure 2: Relative frequency of sero-positive animals by age group. For analysis of age-related
3 effects, 3,965 sera from animals were analysed for antibodies against *C. burnetii* in CHEKIT-Q-
4 Fever ELISA.

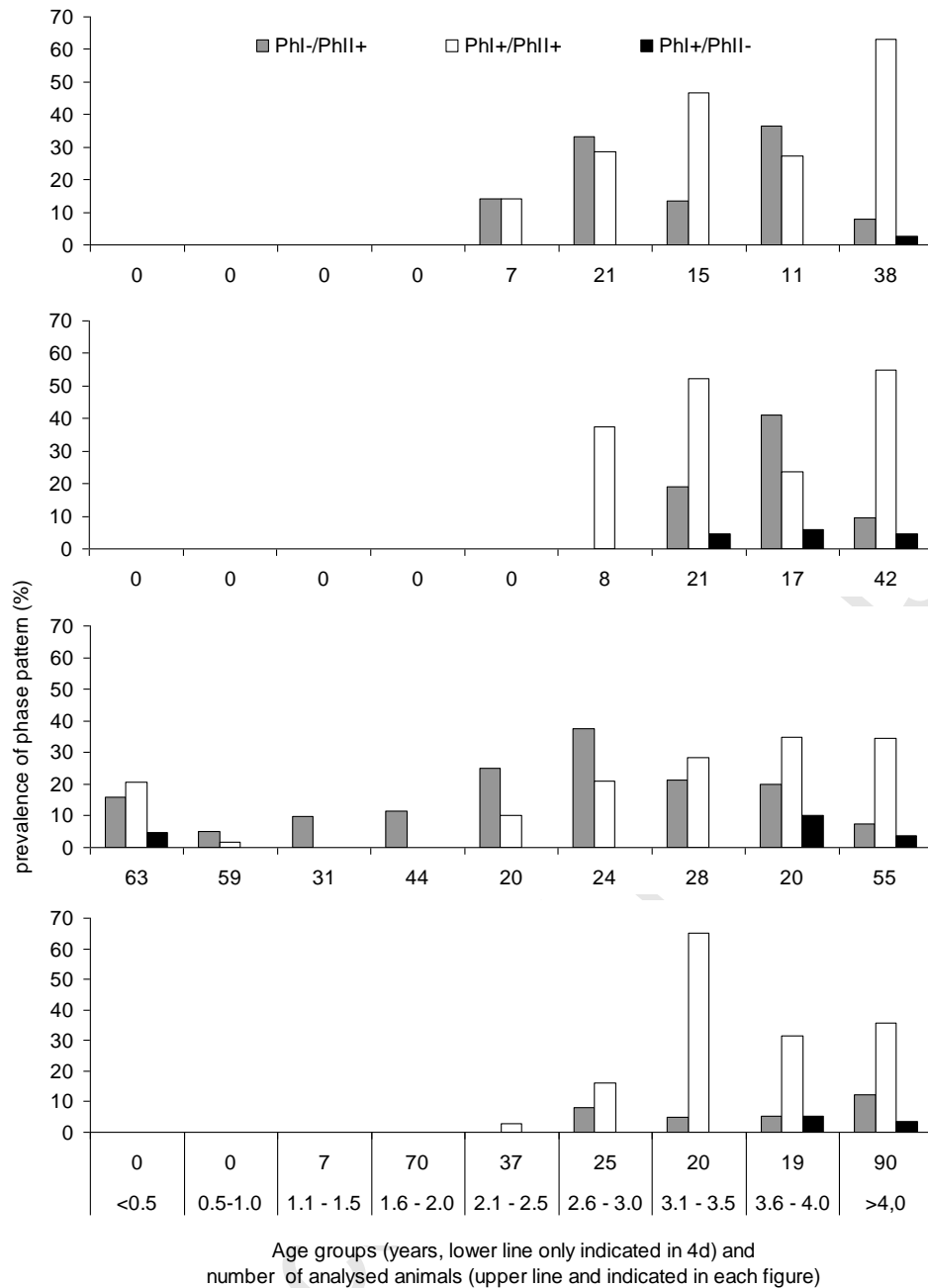
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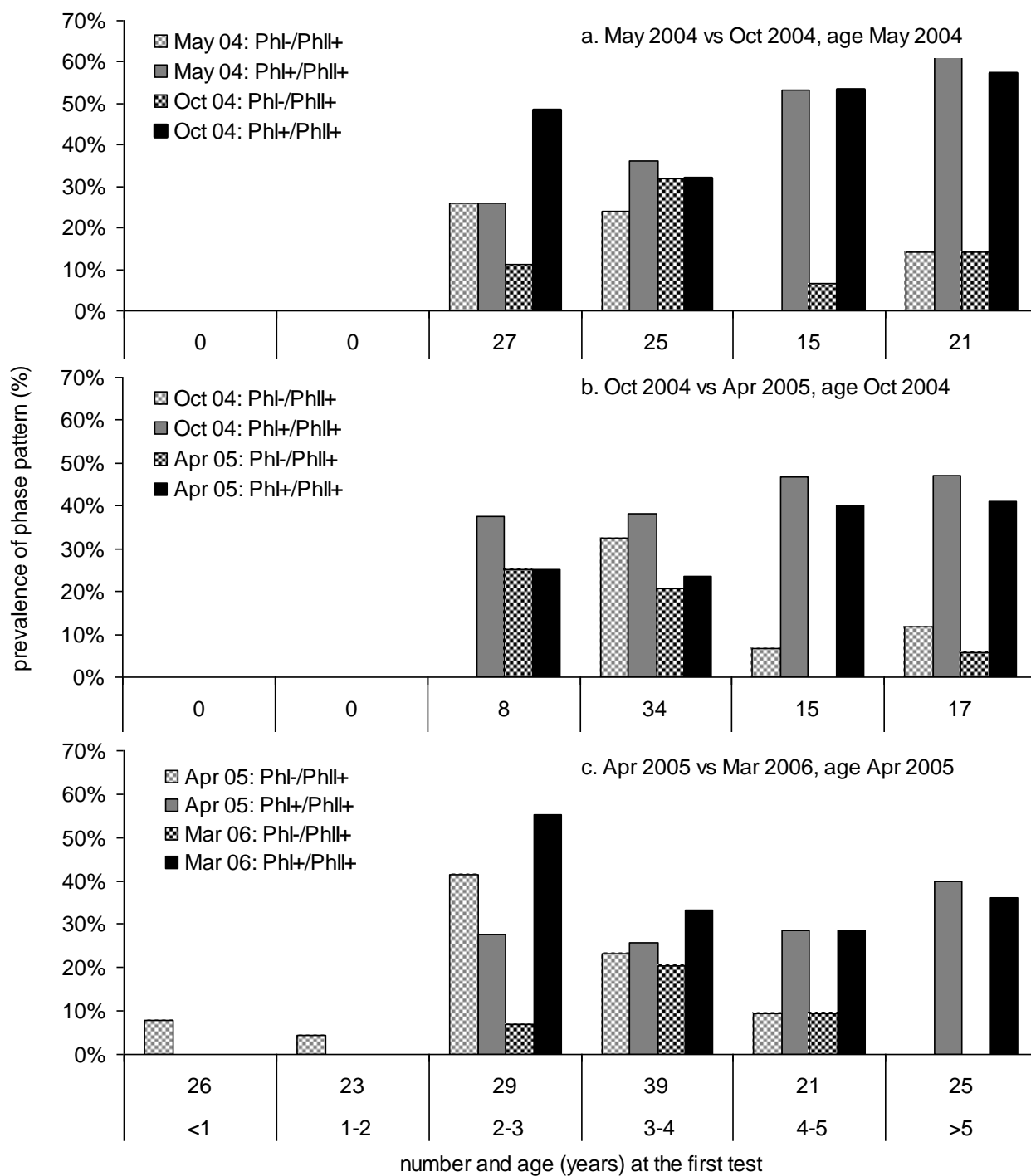


1
2 Figure 3: The cumulative number of PCR-positive placentas over time (month/year) and the
3 number of analysed placentas per month in a dairy herd (herd A) in which *C. burnetii* was
4 detected by PCR in April 2005.

5

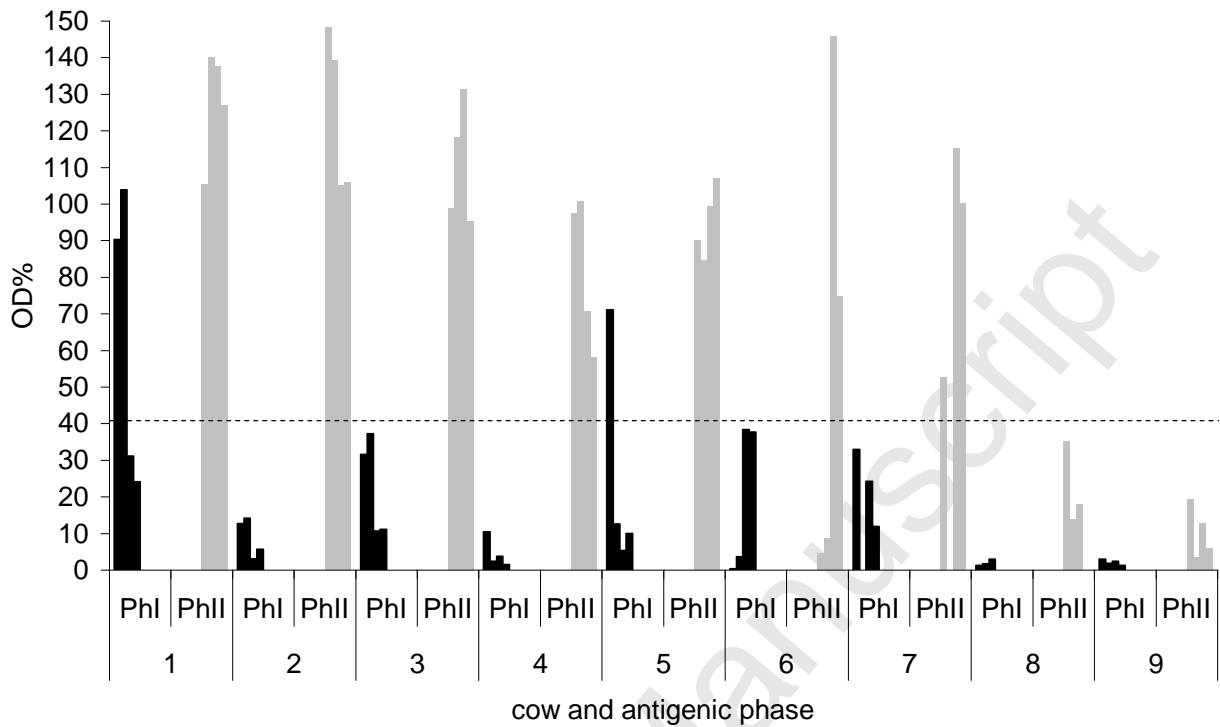


1
 2 Figure 4a-d: In an endemically infected dairy farm, a cross-sectional analysis of sera for
 3 antibodies against *C. burnetii* in phase-specific ELISAs was performed on four occasions: May
 4 (a) and October 2004 (b), April 2005 (c) and March 2006 (d). Prevalences for three possible
 5 phase patterns (PhI⁺/PhII⁺; PhI⁻/PhII⁺; PhI⁺/PhII⁻) were calculated for each age group. Age
 6 groups and the number of analysed animals are indicated on the x-axis of 4d and each figure,
 7 respectively. Note: Not necessarily the same animals are represented in figures 4a-d.; for this it is
 8 referred to Fig. 5a-c.



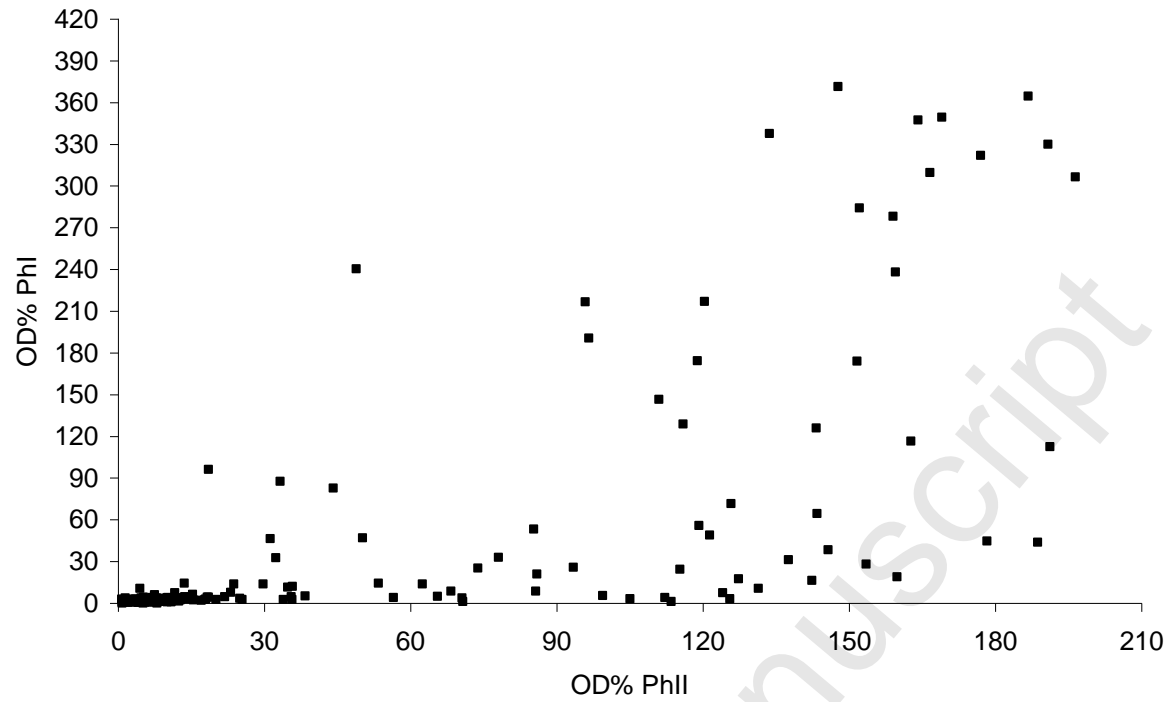
1
 2 Figure 5a-c: Comparison of the prevalence of phase-patterns at two subsequent samplings: Cows
 3 in herd A were sampled in May and October 2004, April 2005 and March 2006. For animals
 4 sampled at two subsequent points in time prevalence of phase pattern was calculated and plotted
 5 over the age at the first respective date of sampling. The number of compared animals per age-
 6 group is indicated beneath each x-axis, whereas age-groups only once are shown at the bottom x-
 7 axis.

1



2

3 Figure 6: Multiparous cows with the PhI/PhII⁺ pattern in Apr 2005 and Mar 2006 (N°1-7) and
 4 two negative cows with PCR-positive results (N°8+9) were analysed for their phase-specific
 5 reactivity over time. Each bar represents a sampling time point: May 2004, October 2004, April
 6 2005, March 2006. No sample was available for cow N° 7 in Oct 2004. A preliminary cut-off of
 7 40% (dashed line) is indicated.



1
2 Figure 7: Comparison of optical densities (OD%) of 164 animals (herd A) for PhI and PhII
3 antibodies in April 2005.

4
5
6