Testing of a palatable bait and compatible vaccine carrier for the oral vaccination of European badgers (*Meles meles*) against tuberculosis

Sonya Gowtage¹, Gareth A. Williams¹, Ray Henderson², Paul Aylett³, Duncan MacMorran³, Si Palmer¹, Andy Robertson^{4, 5}, Sandrine Lesellier¹, Stephen P. Carter^{4,6}, Mark A. Chambers^{1,7}

¹Bacteriology Department, Animal and Plant Health Agency, Addlestone, Surrey KT15 3NB

Email: Sonya.Gowtage@apha.gsi.gov.uk; Gareth.a.Williams@apha.gsi.gov.uk; Si.Palmer@apha.gsi.gov.uk; Sandrine.Lesellier@apha.gsi.gov.uk; Mark.Chambers@apha.gsi.gov.uk

²Pest-Tech Ltd., Branch Drain Road, Brookside, RD2 Leeston, New Zealand. Email: Pest-Tech@hotmail.com

³Connovation Ltd., East Tamaki, Manukau 2013, New Zealand. Email: paul@connovation.co.nz; duncan@connovation.co.nz

Abbreviations: HPO, hydrogenated (hardened) peanut oil; QC, quality control; PT, paste-bait; EP, European pharmacopoeia; USP, United States pharmacopoeia; IPA, iophenoxic acid; TAMC, total aerobic microbial count; TYMC, total yeast and mould count.

22 ⁴National Wildlife Management Centre, Animal and Plant Health Agency, Woodchester
23 Park, Gloucestershire, GL10 3UJ, UK. Email: Andy.Robertson@apha.gsi.gov.uk;
24 Steve.Carter@apha.gsi.gov.uk

25 ⁵Environment and Sustainability Institute, University of Exeter, Penryn, Cornwall, TR10
26 9EZ, UK

27 ⁶*Present address*: The Vincent Wildlife Trust, Ledbury, Herefordshire HR8 1EP. Email:
28 stevecarter@vwt.org

29 ⁷School of Veterinary Medicine, Faculty of Health & Medical Sciences, Vet School Main
30 Building, Daphne Jackson Road, University of Surrey, Guildford, GU2 7AL, UK

31

32 Corresponding author: Dr. Sonya Gowtage, Department of Bacteriology, Animal and Plant
33 Health Agency, Woodham Lane, Addlestone, Surrey KT15 3NB. Email:
34 Sonya.Gowtage@apha.gsi.gov.uk

35 **Abstract**

36 The oral vaccination of wild badgers (*Meles meles*) with live Bacillus Calmette–Guérin
37 (BCG) is one of the tools being considered for the control of bovine tuberculosis (caused
38 by *Mycobacterium bovis*) in the UK. The design of a product for oral vaccination requires
39 that numerous, and often competing, conditions are met. These include the need for a
40 highly palatable, but physically stable bait that will meet regulatory requirements, and one
41 which is also compatible with the vaccine formulation; in this case live BCG. In
42 collaboration with two commercial bait companies we have developed a highly attractive
43 and palatable bait recipe designed specifically for European badgers (*Meles meles*) that
44 meets these requirements. The palatability of different batches of bait was evaluated

45 against a standardised palatable control bait using captive badgers. The physical
46 properties of the bait are described e.g. firmness and colour. The microbial load in the bait
47 was assessed against European and US Pharmacopoeias. The bait was combined with an
48 edible vaccine carrier made of hydrogenated peanut oil in which BCG vaccine was stable
49 during bait manufacture and cold storage, demonstrating $<0.5 \log_{10}$ reduction in titre after
50 117 weeks' storage at 20 °C. BCG stability in bait was also evaluated at +4 °C and under
51 simulated environmental conditions (20 °C, 98% Relative Humidity; RH). Finally,
52 iophenoxic acid biomarkers were utilised as a surrogate for the BCG vaccine, to test
53 variants of the vaccine-bait design for their ability to deliver biomarker to the
54 gastrointestinal tract of individual animals. These data provide the first detailed description
55 of a bait vaccine delivery system developed specifically for the oral vaccination of badgers
56 against *Mycobacterium bovis* using live BCG

57 **Key Words**

58 Badger; BCG; oral vaccination; bait; tuberculosis

59 **1. Introduction**

60 The package of control measures aimed at the eradication of bovine tuberculosis (TB) in
61 England and Wales includes the development of an oral vaccine for badgers (*Meles*
62 *meles*) against the causative agent, *Mycobacterium bovis* [1]. The first injectable vaccine
63 for TB in badgers, BadgerBCG, was licensed in 2010 and has been used in specific areas
64 of England and Wales since then [2,3]. The beneficial effects of the injectable vaccine
65 have been demonstrated in terms of reducing disease severity and progression in captive
66 badgers and reduced serological evidence of infection in wild badgers [4]. The major
67 limitation of BadgerBCG is the need to trap badgers to inject the vaccine. A cost-effective
68 BCG-based oral vaccine could achieve wider coverage, overcoming some of the financial
69 and logistical issues associated with the widespread deployment of BadgerBCG [5]. Oral

70 vaccines against TB are in development for a number of wildlife species besides badgers
71 [6–9]. In all cases, the development and delivery of a licensed oral vaccine product to the
72 field faces many challenges, including effective delivery of the vaccine by consumption,
73 vaccine stability, and environmental safety [10]. These are best exemplified by the
74 comprehensive programme of research and development of the oral rabies vaccine for
75 foxes (*Vulpes vulpes*) in Europe [11], the end product being a ‘tailor-made’ species-
76 specific bait-vaccine product that can be manufactured and deployed at scale. Oral
77 vaccine delivery mechanisms developed for one species are not necessarily appropriate
78 for another, even if the product is palatable. For example, a bait-vaccine carrier developed
79 for wild boar (*Sus scrofa*) was also attractive to badgers in Spain, but of 150 baits
80 consumed by badgers, 87% had the vaccine carriers (plastic capsules) rejected and of
81 these, 99% were separated from the bait intact with the payload of water still inside [12].
82 Here we present the first detailed description of a vaccine delivery system developed
83 specifically for the oral vaccination of badgers with live BCG. Numerous baits and possible
84 vaccine carriers were trialed with both captive and wild badgers. Captive animals were
85 used to screen large numbers of different products of which some were selected for field-
86 testing; only selected data are presented here. The selection of the best bait was
87 dependent on many factors including the potential for ease of manufacture at relatively low
88 cost, as well as the results of associated field studies. The data we present are crucial for
89 the on-going development and eventual licensing of an oral vaccine product for badgers,
90 including: (a) identification and description of important physical characteristics of the bait
91 for potential future quality control (QC) purposes for large-scale manufacture; (b) design of
92 a bait with a compatible and palatable vaccine carrier; (c) evidence that the bait-vaccine
93 carrier design can deliver biomarker to the GIT of badgers as a surrogate for BCG; and (d)
94 evidence that the BCG vaccine remains viable in the delivery system through laboratory
95 production processes, cold-storage and simulated field conditions.

97 2. Materials & Methods

98 2.1 Animals

99 Badgers were either brought in from the wild from TB-free areas under licence, or born in
100 captivity. Wild-caught animals were demonstrated to be free of TB on the basis of IFN γ
101 and clinical sample culture testing and housed in groups (two to five animals per pen) in
102 open-air pens with artificial setts, as described elsewhere [13]. Animals were fed a mixture
103 of commercial dog food, peanuts and occasionally fruit and specified pathogen-free eggs.
104 Each pen was equipped with a motion-sensitive infra-red CCTV camera (Secom Security
105 Systems PLC., Kenley, UK). Groups of two to five penned animals were used in
106 palatability and bait design tests as animals could not be repeatedly housed individually for
107 animal welfare reasons; animals were individually caged for a single night for the
108 biomarker study only. The work was carried out under licences (PL 70/6864 and PL
109 70/7878) from the UK Home Office under the Animal (Scientific Procedures) Act 1986 and
110 approved by the Animal and Plant Health Agency (APHA) Local Ethical Review Panel.

111 2.2 QC of the bait components

112 The bait (referred to as either 'PT' or 'paste bait'), is based on a proprietary recipe and was
113 developed with Pest-Tech Ltd. (Leeston, New Zealand) and Connovation Ltd. (Manukau,
114 New Zealand). The paste is free of anti-microbial preservatives, genetically-modified
115 organisms or animal-derived products. Two physical attributes, namely firmness and
116 colour, were assessed for batches of paste bait post-production. Firmness measurements
117 (kgf) were obtained using a calibrated fruit pressure tester (FT011 with 8 mm tip; ACE
118 Supplies Industrial Ltd., Staplehurst, UK) applied to a minimum of three bait portions (~11
119 g) from each batch. Colour was visually assessed by comparison with a colour chart [14]

120 and the closest match recorded for each batch. Between one and three samples of paste
121 bait from each batch were submitted for microbiological testing (Wickham Laboratories
122 Ltd., Gosport, UK) as soon as possible after manufacture to assess microbial burden
123 against the European pharmacopoeia (EP) and US pharmacopoeia (USP) specifications
124 for 'Non-aqueous preparations for oral use': (a) total aerobic microbial count (TAMC) and
125 total yeast and mould count (TYMC) with limits of $\leq 2 \times 10^3$ and $\leq 2 \times 10^2$ CFU g⁻¹ of bait,
126 respectively; and (b) the absence of *Escherichia coli* in 1 g of material. Where more than
127 one sample was tested per batch of bait, if any one sample exceeded any of the EP or
128 USP specifications, it was considered to have failed QC. Three batches of the vaccine-
129 carrier material, a solid, edible vegetable lipid (HPO, hardened [hydrogenated] peanut oil;
130 Ph. Eur., Sigma-Aldrich Company Ltd., Gillingham, UK) were also submitted for
131 microbiological testing against EP and USP criteria. Palatability testing of the paste bait
132 was carried out between April and October, in both 2013 and 2014, in order to avoid the
133 winter months when badgers exhibit reduced activity [15] and to correspond to when oral
134 bait vaccine would most likely be deployed in the field. In each test (one test per batch of
135 bait) between six and eight groups of animals were each presented with bait contained in
136 five litre plastic tubs (two white and two grey), which were placed in a Latin square
137 arrangement in each pen (tubs approx. 1 m from each other) in the late afternoon before
138 the animals emerged from their setts. Two tubs contained 400 ± 1 g of a batch of paste
139 bait each and two tubs contained 400 ± 1 g of control bait each, comprising a mix of
140 peanuts and golden syrup (ratio 8:1), known to be highly palatable to badgers [16]. Tests
141 were run overnight and normal feed was either withheld for the entire night or given to a
142 group after they had consumed some, but not all, of either bait type; water was provided
143 ad libitum. Limited consumption of bait by a group of animals (i.e. <20 g of one or both bait
144 types, as recommended by the manufacturer) was not considered to be representative of a
145 definitive preference and could result in incorrect palatability calculations. Therefore

146 groups which consumed <20 g of material were not included in the palatability
147 calculations. Palatability (%) was calculated for each group of animals by dividing the
148 weight of test bait consumed by the total quantity of bait (test and control) consumed by
149 the group. The final palatability value for a batch of bait was calculated by taking an
150 average across all groups. The peanut and syrup control provided a benchmark for
151 palatability, as a minimum standard for a palatable bait. Therefore any test material was
152 required to be at least as palatable as the control i.e. have a palatability of $\geq 50\%$.
153 However, a more stringent palatability threshold of 65% was set for this work to allow for
154 the greater variability introduced when using a small number of groups for testing; ideally
155 palatability tests would utilise large numbers of individually caged animals (R. Henderson,
156 Pest-Tech Ltd., personal communication).

157 *2.3 Bait design tests: bait consumption*

158 Variants of the PT-HPO bait design (Fig. 1) were evaluated in two tests designed to
159 investigate whether altering the PT:HPO ratio and varying the surface area of the exposed
160 PT (to enhance odour release) affected bait disappearance and/or preference. Groups of
161 captive animals were presented with bait portions under inverted terracotta plant pots (with
162 a drainage hole in the base) placed on saucers. Baits, with each type under a different pot,
163 were placed in the pens in the late afternoon prior to the animals emerging from their setts.
164 The delivery of normal feed was delayed until 23:00 each night, to give the animals time to
165 interact with the baits with no other food present. Infra-red CCTV footage of the night was
166 then reviewed to determine (a) the numbers of baits taken - whether or not the bait was
167 consumed in view of the cameras, and (b) preference as measured by the order in which
168 different baits were taken (mean order baits taken across all groups). The peanut and
169 syrup mix was prepared as described previously and presented in portions equal in weight
170 to the baits presented in each pen. In test 1, three different designs of the PT-HPO bait
171 ('SH', 'M' and 'C', Fig. 1), ranging in weight from 13 g to 16 g each, were presented to ten

172 groups of animals (between three and five animals per group) over two nights in three-
173 choice tests. The different baits (one bait of each type per group) were rotated through
174 each pen and each position, left to right, in view of the camera. Test 2 included the bait
175 designs from test 1 and an additional two designs ('T' and 'D', Fig. 1) in a four-choice test.
176 Bait designs were rotated through each group over three nights such that each potential
177 bait combination was presented to each group.

178 2.4 Bait design tests: biomarker delivery

179 Two different iophenoxic acid (IPA) biomarkers (PR euroCHEM Ltd., Cork, Ireland) were
180 incorporated into each of two bait designs (selected from those tested in tests 1 and 2
181 above) in order to determine whether they could, irrespective of packaging type, deliver
182 IPA to the GIT as a surrogate for the BCG vaccine. Each bait contained 80 ± 4 mg ($\pm 5\%$)
183 of Propyl-IPA powder (P-IPA; a-propyl,b-(3- hydroxy-2,4,6-triiodo)phenyl-propionic acid)
184 weighed directly into the centre of the bait within the HPO core in place of the BCG.
185 Isobutyl-IPA (I-IPA; a-isobutyl,b-(3-hydroxy-2,4,6-triiodo)phenyl-propionic acid) was
186 incorporated by evenly dissolving 80 mg throughout the HPO component to provide
187 internal calibration for the amount of actual vaccine carrier consumed. A total of 20
188 animals were presented with a single bait each and bait allocation was randomised. Ten
189 were given the PT-HPO 'M' in a greaseproof paper bag (approx. 140 x 170 mm,
190 Connovation Ltd.) and 10 were given the 'SH' bait packaged in a low density polyethylene
191 (LDPE)-lined paper bag (approx. 90 x 170 mm). Different types of packaging were being
192 evaluated in order to inform packaging field tests [17]. Materials such as plastic-lined
193 paper may offer increased protection to the bait against moisture, relative to a paper bag.
194 Individual animals were trapped overnight in cages with a single bait for one night only;
195 water was provided in a bowl. Animals were released the following morning and any bait
196 fragments found in the cages collected and weighed. Only animals that consumed some or
197 all of the bait were anaesthetised by an intramuscular injection of ketamine and

198 medetomidine (10 mg/kg; Vetalar[®] and 100 µg/kg; Domitor[®], respectively; Pfizer Animal
199 Health, New York, USA) and bled by jugular venepuncture into Serum Separation Tubes
200 (SST Vacutainer tubes; BD, Plymouth, UK) 13–15 days after bait consumption, to coincide
201 with peak IPA serum levels (based on unpublished findings). Serum samples were stored
202 frozen (80 °C) and submitted for duplicate analysis by liquid chromatography-mass
203 spectrometry (LC-MSMS; LGC, Teddington, UK), to quantify and differentiate between P-
204 IPA and I-IPA.

205 *2.5 Stability of BCG in PT-HPO baits*

206 Following successful additional field testing as a carrier (K. Palphramand *et al.*,
207 unpublished results), and on the basis of ease of manufacture, the ‘SH’ and ‘M’ PT-HPO
208 baits (Fig. 1) were used to test vaccine stability. A 200 µl volume of BCG vaccine
209 suspension (Danish strain 1331; ‘Concentrated Bulk BCG’ preparation, Statens Serum
210 Institute, Copenhagen, Denmark), was encapsulated within the centre of the HPO (Fig.
211 1b). BCG stability (survival) within baits was evaluated at the storage temperatures of 20
212 °C and 4°C without humidity control, and at 20 °C at 98% relative humidity (RH)
213 maintained using an environmental test chamber (THC Slimline 600/800/-40/ME;
214 Sharetree Ltd., Stonehouse, UK). The conditions in the environmental chamber were
215 based on RH measurements taken from badger setts by LogTag[®] recorders (HAXO-8;
216 LogTag Recorders Ltd., Auckland, New Zealand) during a Spring-Summer bait
217 deployment study (unpublished results) and from UK MET Office soil temperatures, as
218 indicative of the conditions a product would experience if deployed down a sett entrance.
219 BCG was extracted from baits at regular intervals by cutting each bait into small fragments
220 to release the BCG from the centre. The fragments were submerged in Sauton liquid
221 medium supplemented with 0.05% (v/v) Tween 80 and agitated to macerate the PT and
222 wash the BCG from the fragments in order to form a suspension. Aliquots of this

suspension and dilutions thereof were plated on Modified 7H11 agar plates and BCG colonies enumerated after four weeks' incubation at 37 °C. Log change values were calculated as $\log_{10}$ (CFU per bait) at each sampling point minus the value at the first time point for the storage conditions under evaluation.

2.6 Statistical analyses

Chi-squared tests were used to test whether badgers exhibited evidence of preference for particular bait types (measured as the order in which baits were consumed), or whether they simply selected different bait types at random. Correlations between mean P-IP and mean I-IPA values of individual badgers were tested using Pearson correlation tests. Chi-square and correlation analyses were carried out using R 3.0.2 (cran.org). The numbers of badgers consuming 'M' and 'SH' bait types were compared using Fisher's Exact Test (two-tailed) performed in GraphPad Prism[®] 6 software (GraphPad Software, Inc., USA).

3. Results

3.1 QC of the bait components

The firmness of the paste bait ranged between 1.8 and 3.2 kgf with a mean of 2.3 kgf (SEM 0.04 kgf; n = 87 tests for 16 batches of bait). Of these 16 batches, two had TAMC in excess of 2×10^3 CFU g⁻¹ but none had TYMC above the limit of 2×10^2 CFU g⁻¹; *E. coli* was not detected in any of the samples. The three batches of HPO were all free of *E. coli* and all had TAMC of $<1 \times 10^1$ CFU g⁻¹ and TYMC of $\leq 5.0 \times 10^0$ CFU g⁻¹. The colour of the paste bait was consistent between batches. Thirteen batches of paste bait were palatability tested and each batch was presented to 6–8 groups of animals. Overall mean palatability was 77% (67–87%) with none of the batches having a mean palatability of less than the 65% target threshold (Fig. 2).

3.2 Bait design tests

247 The percentage of baits taken in tests 1 and 2 was high for all designs (80–100%) and the
248 order of bait selection was not significantly different from that expected if animals were
249 selecting at random (Chi-squared tests, test 1 : $\chi^2_9 = 6.67$, $p = 0.67$, test 2: $\chi^2_9 = 8.58$, $p =$
250 0.37). Of the bait designs, 'SH' and 'M' (Fig. 1), were selected for the biomarker study
251 based on size, handling by the animals and ease of manufacture. The number of individual
252 badgers consuming bait was 8/10 and 5/10 for the 'M' and 'SH' baits respectively (NS
253 difference, Fisher's Exact Test). Very few fragments were recovered post-consumption
254 with their weight constituting 2.1% ($n = 3$) and 2.4% ($n = 3$) of the 'M' and 'SH' baits
255 respectively. Every animal that consumed a bait had positive serum-IPA levels and mean
256 serum values for P-IPA and I-IPA were closely correlated for the bait designs under
257 evaluation (Pearson correlation, $t_{25} = 6.98$, $p < 0.001$, $r = 0.81$).

258 3.3 BCG stability in PT-HPO baits

259 The oral BCG dose required to successfully vaccinate badgers in the field is not known. In
260 addition, storage, distribution and deployment of a commercial oral badger vaccine is likely
261 to expose BCG-containing baits to a range of temperatures and for varying durations. For
262 these reasons, the stability of BCG in bait was broadly characterised using a range of
263 different starting concentrations, temperatures and durations: 2.90×10^6 – 1.32×10^8
264 CFU/bait at 20 °C, 1.24×10^7 – 9.20×10^7 CFU/bait at 4 °C and 5.40×10^7 – 6.20×10^7
265 CFU/bait at 20°C and 98% RH. BCG-containing baits evaluated at 4°C and 20 °C 98% RH
266 had previously been stored at 20°C for between 4 and 207 days, and 25 days,
267 respectively. BCG retained viability in bait best when stored at 20 °C (Fig. 3a) with <0.5
268 $\log_{10}$ decrease in CFU/bait up to 117 weeks storage. BCG was less stable in bait when
269 stored at 4 °C or at 20 °C, 98% RH, with notable and more rapid decreases in CFU/bait
270 becoming apparent after approximately 7 days' storage (Fig. 3b and c). The overall
271 physical structure of the vaccine-baits remained intact over a 30-day period (Fig. 4).

272 4. Discussion

273 The data presented here fulfil the first important steps in the development and testing of a
274 tailor-made oral vaccine product for UK badgers against TB, namely palatability, vaccine
275 stability during bait manufacture and storage, determination of objective and descriptive
276 criteria for QC, and demonstration of its ability to deliver biomarker after ingestion as a
277 surrogate for the vaccine.

278 The data generated from the QC of the paste bait not only allowed for a better definition of
279 the product but also demonstrated, for the majority of the batches tested, a consistent
280 standard of manufacture. This is important for future bait production but also reassuring
281 given the nature of some of the key bait ingredients which are plant-derived and
282 preservative-free and therefore more variable in nature than, for example, more highly
283 processed products such as the HPO component that will carry the vaccine. Slight
284 variations in paste-bait consistency and colour did not affect its palatability to captive
285 animals as the material was consistently more palatable (on average, more paste bait was
286 consumed than control bait in all batch tests; Fig. 2) to captive animals than the peanut-
287 syrup mix, which is known to be highly attractive to badgers. There are unavoidable
288 limitations associated with the use of captive animals for bait tests, namely the limited
289 sample size and the possible effects of an artificial diet on bait preference and
290 consumption. However, promising materials were always field-tested and these tests
291 reflected the high attractiveness and palatability of the PT-HPO baits observed with
292 captive animals [17].

293 The paste bait component fulfils several important roles as an effective lure (release of
294 olfactory cues), a palatable material and as an adaptable and malleable material
295 compatible with a vaccine carrier (in this case HPO). Smell is a very important sense for
296 badgers [18] and so the release of odour by the paste bait is likely to be important for

297 attracting individuals to the bait which is why the bait designs (Fig. 1) varied in the surface
298 area of exposed PT (that not covered by the HPO). The PT component may also help to
299 mask the taste of the vaccine payload; PT-HPO baits containing 200 µl of BCG have
300 proven palatable (88% and 92% of baits taken) with individually caged badgers in two
301 vaccine efficacy trials (S. Lesellier, personal communication). In the current study,
302 variations in bait design did not appear to affect attractiveness (percentage of baits taken)
303 allowing bait design to be driven by other criteria including ease of manufacture, cost (as
304 determined by the quantities of the ingredients) and physical stability; bait size did not
305 affect preference as baits of different sizes were also readily accepted by wild badgers [17]
306 (K. Palphramand et al., Unpublished results).

307 The use of the IPA biomarkers demonstrated that irrespective of bait design, a core of
308 biomarker could be ingested yielding a positive serum sample in every individual that
309 consumed bait, thus complementing IPA-data from a vaccine carrier field study (K.
310 Palphramand et al., unpublished results). In addition, very little of each bait was left behind
311 in the cages of badgers which had consumed baits, which mirrors observations in the field
312 that animals generally eat any bait fragments which fall from the mouth during
313 consumption (K. Palphramand, personal communication); removal of bait fragments by
314 badgers would also limit consumption by non-target species.

315 HPO fulfils many requirements of a vaccine carrier: (1) it is palatable, whether presented
316 alone (S. Gowtage, unpublished results) or with the paste bait; (2) it provides good
317 adherence and structural compatibility with the paste bait; (3) it provides an environment
318 for long-term stability of BCG at 20°C; and (4) it is solid over the range of temperatures
319 likely to be encountered in the field (HPO Ph. Eur. drop point specified range 32–43 °C).

320 **5. Conclusions**

321 We have developed an oral bait-vaccine delivery system suitable for further evaluation
322 with captive and wild badgers. Many of these tests are already underway, including
323 evaluation of the efficacy of oral BCG fed to captive badgers in the context of PT-HPO and
324 devising a pragmatic protocol for deployment of PT-HPO to wild badgers to maximise bait
325 uptake. Future work will also comprise the necessary technology transfer for larger-scale
326 manufacture. BCG was stable in the bait during prolonged periods of storage frozen. BCG
327 viability declined at 4 °C and 20 °C but not appreciably until seven days at either
328 temperature, indicating that BCG may retain sufficient viability during cold storage and field
329 deployment to make it a viable oral vaccine. However, further studies will be required once
330 the minimum efficacious dose of the vaccine is determined.

331

332 **Acknowledgements**

333 This work was funded by the Department for Environment, Food and Rural Affairs (Defra).
334 The authors would like to thank the Animal Services Unit staff and colleagues in the
335 Immunology and Vaccines Work Group at APHA for their help. The authors also thank K.
336 Palphramand for her input.

337 **Conflict of interest statement**

338 Conflicts of interest: none

339

340

341 **References**

- 342 [1] Department for Environment, Food & Rural Affairs. Bovine TB eradication
343 programme for England. London: Defra; 2011. p. 64.
- 344 [2] Department for Environment, Food & Rural Affairs. The strategy for achieving
345 officially bovine tuberculosis free status for England. England: Defra; 2014. p.85.
- 346 [3] Welsh Government. Badger vaccination in the intensive action area; 2016
347 [http://wales.gov.uk/topics/environmentcountryside/ahw/disease/
348 bovinetuberculosis/intensive-action-area/badger-vaccination-iaa/?lang=en](http://wales.gov.uk/topics/environmentcountryside/ahw/disease/bovinetuberculosis/intensive-action-area/badger-vaccination-iaa/?lang=en) [accessed
349 03.02.16].
- 350 [4] Carter SP, Chambers MA, Rushton SP, Shirley MD, Schuchert P, Pietravalle S,
351 et al. BCG vaccination reduces risk of tuberculosis infection in vaccinated
352 badgers and unvaccinated badger cubs. PLoS ONE 2012;7:e49833.
- 353 [5] Robinson PA, Corner LA, Courcier EA, McNair J, Artois M, Menzies FD, et al. BCG
354 vaccination against tuberculosis in European badgers (*Meles meles*): a review.
355 Comp Immunol Microbiol Infect Dis 2012;35:277–87.
- 356 [6] Beltran-Beck B, Romero B, Sevilla IA, Barasona JA, Garrido JM, Gonzalez-Barrio
357 D, et al. Assessment of an oral *Mycobacterium bovis* BCG vaccine and an
358 inactivated *M. bovis* preparation for wild boar in terms of adverse reactions,
359 vaccine strain survival, and uptake by nontarget species. Clin Vac Immunol 2014;21:12-
360 20.
- 361 [7] Corner LA, Costello E, O'Meara D, Lesellier S, Aldwell FE, Singh M, et al. Oral
362 vaccination of badgers (*Meles meles*) with BCG and protective immunity
363 against endobronchial challenge with *Mycobacterium bovis*. Vaccine 2010;28:6265–72.
- 364 [8] Palmer MV, Thacker TC, Waters WR, Robbe-Austerman S. Oral vaccination of
365 white-tailed deer (*Odocoileus virginianus*) with *Mycobacterium bovis* Bacillus
366 Calmette-Guerin (BCG). PLoS ONE 2014;9:e97031.
- 367 [9] Tompkins DM, Buddle BM, Whitford J, Cross ML, Yates GF, Lambeth MR, et al.
368 Sustained protection against tuberculosis conferred to a wildlife host by single

369 dose oral vaccination. *Vaccine* 2013;31:893–9.

370 [10] Chambers MA, Carter SP, Wilson GJ, Jones G, Brown E, Hewinson RG, et al.
371 Vaccination against tuberculosis in badgers and cattle: an overview of the
372 challenges, developments and current research priorities in Great Britain. *Vet Rec*
373 2014;175:90–6.

374 [11] Pastoret P-P, Kppeler A, Aubert M. European rabies control and its history. In:
375 King AA, Fooks AR, Aubert M, Wandeler AI, editors. *Historical perspective of*
376 *rabies in Europe and the Mediterranean Basin*. Paris, France: World
377 Organisation for Animal Health (OIE); 2004. p. 337–50.

378 [12] Ballesteros C, Vicente J, Morriss G, Jockney I, Rodriguez O, Gortazar C, et al.
379 Acceptance and palatability for domestic and wildlife hosts of baits designed
380 to deliver a tuberculosis vaccine to wild boar piglets. *Prev Vet Med* 2011;98:198–203.

381 [13] Lesellier S, Palmer S, Gowtage-Sequeira S, Ashford R, Dalley D, Davé D, et al.
382 Protection of Eurasian badgers (*Meles meles*) from tuberculosis after intramuscular
383 vaccination with different doses of BCG. *Vaccine* 2011;29:3782–90.

384 [14] Wikipedia. List of colors: A–F.
385 http://en.wikipedia.org/wiki/List_of_colors:_A%E2%80%93F [accessed 2013].

386 [15] Roper TJ. Basic biology. In: Corbet SA, West R, Streeter D, Flegg J, Silvertown J,
387 editors. *Badger*. London: Collins; 2010. p. 43–80.

388 [16] Palphramand KL, Walker N, McDonald RA, Delahay RJ. Evaluating seasonal bait
389 delivery to badgers using rhodamine B. *Eur J Wildlife Res* 2011;57:35–43.

390 [17] Robertson A, Delahay RJ, McDonald RA, Aylett P, Henderson R, Gowtage S, et al.
391 Behaviour of European badgers and non-target species towards candidate
392 baits for oral delivery of a tuberculosis vaccine. *Prevent Vet Med* 2016;135:95–101.

393 [18] Neal E, Cheeseman C. General characteristics. *Badgers*. London: T & AD Poyser
394 Ltd.; 1996.

395 **Fig. 1.** Different designs of the PT-HPO bait. As referred to in the text – ‘SH’, ‘M’, ‘C’, ‘T’
396 and ‘D’; b) Vertical (top) and horizontal (bottom) sections of a frozen ‘M’ bait showing the
397 location of the 200µl vaccine payload (arrows). Bar = 1 cm. All baits comprised the same
398 basic structure in which the vaccine payload was held centrally in the HPO (white lipid);
399 the IPA was located in the same position for the biomarker study. The HPO containing the
400 vaccine was surrounded by a PT cylinder (brown paste bait) which in the case of the ‘SH’,
401 ‘M’ and ‘C’ baits was then in turn surrounded by an external layer of HPO.

402 Abbreviations: PT, paste bait; HPO, hardened peanut oil; IPA, iophenoxic acid.

403
404

405 Fig. 2. Mean palatability (%) for each batch of paste bait (n=13). Each bar represents the
406 mean palatability for a batch of bait and error bars indicate the range in palatability for
407 each batch. Between 4-6 groups of animals (mean 5.2) consumed bait (>20 g) in each
408 batch test. The dashed line is the cut-off of 65% below which a batch of bait was
409 considered to be insufficiently attractive and palatable by the manufacturer.

410

411

412 **Figure 3:** Stability profiles of BCG in vaccine-baits stored under different conditions: (a) -
413 20 °C, (b) 4 °C and (c) 20 °C 98% relative humidity. Data points are the calculated mean
414 $\log_{10}$ change in CFU/vaccine-bait relative to the mean dose determined before incubation
415 under the conditions stated. The numbers of vaccine-baits sampled at each point are
416 indicated above the range error bars (where visible). Vaccine-baits evaluated at 4 °C and
417 20 °C 98% RH (relative humidity) had previously been stored at -20 °C for between 4 and
418 207 days, and 25 days, respectively.

a

b

c

420 **Fig. 4.** Photographs of packaged BCG-PT-HPO vaccine-baits stored at 20 °C and 98% RH
421 for (a) 2 days and (b) 30 days. There was only limited absorption of water by the exposed
422 PT component on the surface of the bait (area indicated by the scalpel in 'b').

423 Abbreviations: PT, paste bait; HPO, hardened peanut oil; RH relative humidity.