Veterinary Parasitology 267 (2019) 42–46

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

A novel, high-welfare methodology for evaluating poultry red mite interventions *in vivo*

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ARTICLE INFO

Keywords:
Poultry red mite
Dermanyssus gallinae
Feeding device
3Rs

ABSTRACT

Optimisation and use of a device for the on-hen *in vivo* feeding of all hematophagous stages of *Dermanyssus gallinae* is described. The sealed mesh device contains the mites and is applied to the skin of the hen's thigh where mites can feed on the bird through a mesh which has apertures large enough to allow the mites' mouthparts to access to the bird but small enough to contain the mites. By optimising the depth and width of the mesh aperture size we have produced a device which will lead to both reduction and refinement in the use of animals in research, allowing the pre-screening of new vaccines and systemic acaricides/insecticides which have been developed for the control of these blood-feeding parasites before progressing to large field trials. For optimal use, the device should be constructed from 105 µm aperture width, 63 µm depth, polyester mesh and the mites (irrespective of life stage) should be conditioned with no access to food for 3 weeks at 4 °C for optimal feeding and post-feeding survival.

1. Introduction

Infestation of laying hen houses with poultry red mites (*Dermanyssus gallinae*) causes major animal welfare and economic losses for the egg-producing industry worldwide, costing in excess of €231 million per year in the European Union alone in control and production losses (Sigognault Flochlay et al., 2017). Poultry red mites are blood-feeding ectoparasites and can form large populations in the accommodation of birds kept for long periods of time (˜1 year) such as those found in commercial egg laying operations. Only the eggs and larvae of the mites are non-feeding, with the protonymph, deutonymph and adults all feeding on blood by biting the hens during the hours of darkness. Moderate infestations of mites (approximately 50,000 parasites per hen), can impact on the welfare of the birds, inducing behaviours such as increased restlessness, feather pecking and cannibalism whereas in severe infestations (500,000 mites per hen) substantial welfare issues such as anaemia and death are seen as well as losses in production (Van Emous, 2005: Sparagano et al., 2014). In addition to the direct effects of infestation, *D. gallinae* is also increasingly being recognised as a competent vector for pathogens that cause a number of important avian and zoonotic diseases, including *Salmonella* Enteritidis (Sparagano et al., 2014) and avian influenza virus (Sommer et al., 2016) and can serve as a reservoir for fowl typhoid between sequential hen flocks (Pugliese et al., 2018).

Demand for novel methods of control is driven by the inadequacy of many current chemotherapeutic control methods which results in uncontrolled infestations and substantial welfare issues and commercial losses. The recent surge in scientific research activity for novel methods of controlling poultry red mites has encompassed novel biopesticides and plant derived products; semiochemicals and growth-regulators; vaccines; biological control; physical barriers; architectural, engineering and process-management solutions (Sparagano et al., 2014; Sigognault Flochlay et al., 2017).

Typically, the testing of many of these new interventions uses small numbers of mites in *in vitro* efficacy assays initially, followed directly by field testing using large numbers of both parasites and hens (e.g. Bartley et al., 2015; Wright et al., 2016; Bartley et al., 2017; Thomas et al., 2018). When applied to novel interventions being delivered to the mites via the hen's blood (e.g. novel vaccines and systemic acaricides), this strategy has some utility but suffers from highly variable mite feeding rates and high background mortality of mites when using the *in vitro* feeding system (Bartley et al., 2015; Wright et al., 2016). In addition,

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https://doi.org/10.1016/j.vetpar.2019.01.011
Received 24 October 2018; Received in revised form 7 January 2019; Accepted 12 January 2019

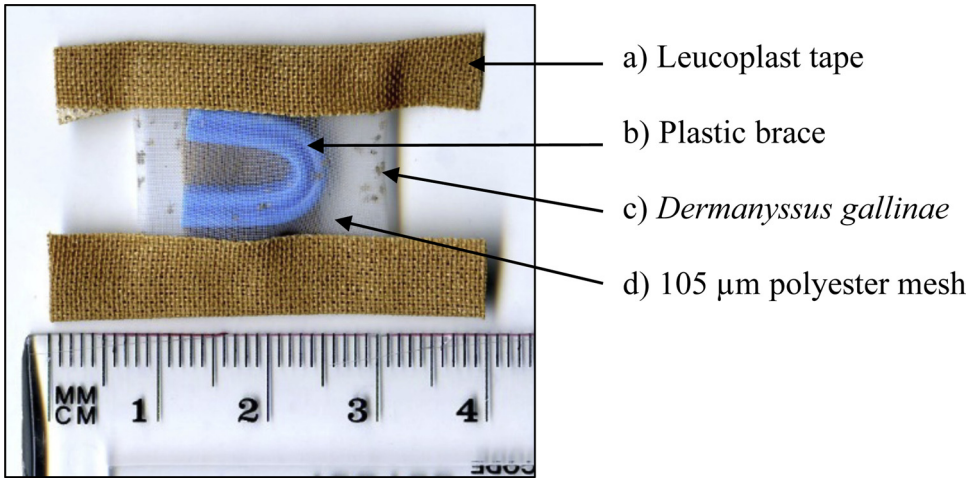

Fig. 1. Design of the optimised on-hen *in vivo* feeding device.

our previous studies have shown that vaccine efficacy measured using the *in vitro* feeding device is not always translated into mite population reduction in the field trials (Bartley et al., 2017).

To address these issues an 'on-hen' *in vivo* mite feeding device could be used as an alternative to the *in vitro* feeding assays for more accurate pre-screening of potential novel interventions before embarking on field studies. Previously, a rigid prototype device for containing mites on hens was developed (Harrington et al., 2010) but this has not been widely adopted, possibly due to the issues around attachment and access of mites to feed on the skin when the feeding device is inflexible. In the work we describe here, we therefore developed and optimised a flexible on-hen *in vivo* feeding device using different mesh aperture dimensions and mite conditioning protocols to allow all haemophagous life stages of *D. gallinae* to efficiently feed on host hens in a contained and reproducible manner.

2. Materials and methods

2.1. Hens

Lohman Brown pullets (18-weeks-old) were housed in an enclosed, loose litter floor pen of 2.5 m x 2 m with temperature (18 °C) and lighting (16 h light/8 h dark) controlled to mimic commercial hen laying conditions. Hens were in good condition with no evidence of feather damage which would indicate previous exposure to *D. gallinae*. Hens had *ad libitum* access to layers pellets and water; perches and nest boxes were provided.

2.2. Parasite material

For provision of parasite material, mixed stage and sex *D. gallinae* were collected weekly from a commercial egg laying unit. Adult females and deutonymphs were isolated from the mite pool whereas protonymphs were provided by hatching mite eggs and allowing the emerging larvae to moult over a 3 day period at 25 °C and 85% relative humidity (RH). Prior to feeding assays, mites were stored in vented 75 cm^2 canted tissue culture flasks (Corning, NY, USA) at room temperature (RT) for seven days, after which they were stored at 4 °C for 0–3 weeks, dependent on the specific experiment.

2.3. Feeding device manufacture and optimisation

To optimise the mesh size for the feeding device, 50 adult female mites, along with 50 deutonymphs or 50 protonymphs were sealed into feeding devices constructed from the different meshes. Nylon phytoplankton mesh with aperture widths of 75 µm (pore depth ~150 µm), 125 µm (pore depth ~170 µm) (Marine Aquafarm, Thornton-Cleveleys, UK) and 120 µm (pore depth 82 µm) (Plastok Ltd, Birkenhead, UK) and polyester mesh with aperture widths of 68 or 105 µm (pore depths 45 µm and 63 µm respectively) (Plastok Ltd, Birkenhead, UK) were assessed. Feeding devices (Fig. 1) were assembled by making a tube from a strip of mesh 6 cm x 30 cm and sealing the long edge together with Leucoplast tape (BSN Medical) on the inner surface of the mesh tube. A strip of AeraSeal tape (Sigma-Aldrich Co Ltd, Dorset, UK) was then placed on the outer surface of the long edge, covering any exposed parts of the adhesive surface of the Leucoplast tape. The resulting tube was cut into 3 cm segments and one end of each sealed on the outside edge with Leucoplast tape to form a pouch. Plastic braces constructed from a 2.5 cm cut section of a disposable plastic QuadLoop (Sterilin, ThermoFisher Scientific, UK) and gently heated and bent into a 'U' shape were placed inside each pouch to keep the mesh sides apart and to provide the mites with refuge. Mites were placed into the feeding devices and the open end sealed on the outside edge with a strip of Leucoplast tape (Fig. 1) Mites were placed into the devices on the day before each feeding assay and stored overnight at ~10 °C.

2.4. Feeding assays and mite recovery

Prior to the first assay, feathers were plucked from an area of the outer thigh of each hen and thereafter whenever needed. Feeding devices (1 per thigh) containing mites were placed onto the plucked thighs of the hens and secured with Leucoplast tape (BSN Medical, Germany), ensuring that the mesh surfaces were not obscured. The device was further secured with cohesive bandage (Central Medical Supplies Ltd., Pontefract, UK) wrapped over the feeding device which not only secures the device on to the hen but also provides dark conditions for mite feeding. Feeding devices were left in place for 3 h, during which time hens were allowed to carry out normal foraging and nesting behaviours. Following the feeding assay, the devices were removed and fully engorged and partially fed mites were recovered from the devices and placed individually into wells of 96 well tissue culture plates (Costar, Corning, NY, USA), sealed with AeraSeal tape (Sigma-Aldrich Co Ltd, Dorset, UK) and incubated at 25 °C with 85% RH and monitored for mortality at 3, 48, 96 and 144 h.

2.5. Optimisation of mite feeding

To determine the optimal mesh size for feeding, eight hens each wore two pouches containing 50 adult females and 50 of either deutonymphs or protonymphs. The 75 µm and 125 µm aperture nylon meshes and the 68 µm aperture polyester mesh were assayed once and then discarded. The 120 µm and 105 µm aperture polyester meshes

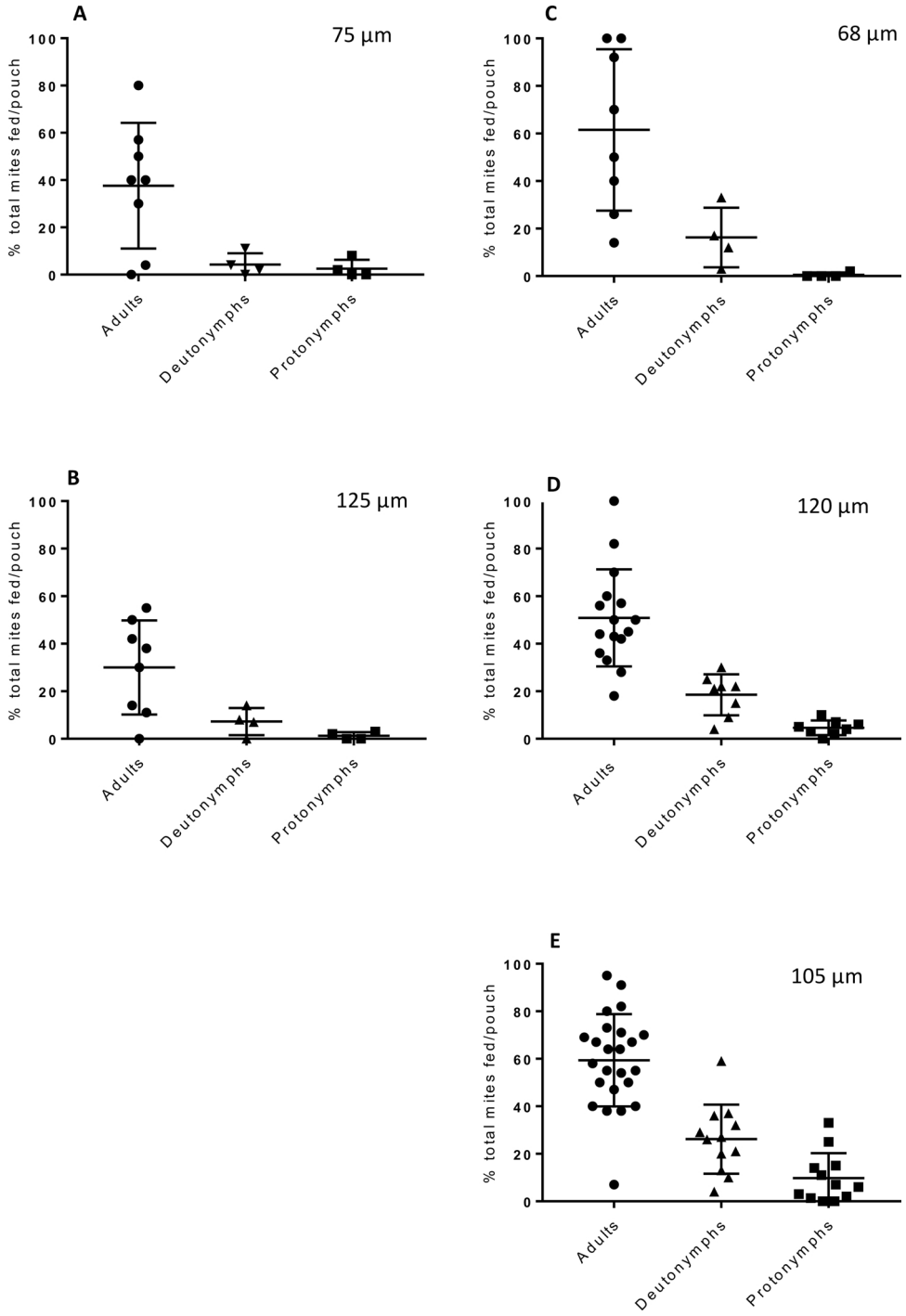

Fig. 2. The ability of hematophagous life stages of *Dermanyssus gallinae* to take a blood meal from hens through nylon meshes with aperture dimensions of 75 µm wide by 150 µm deep (Panel A) and 125 µm wide by 170 µm deep (Panel B) and polyester mesh with aperture dimensions of 68 µm by 45 µm deep (Panel C), nylon mesh with aperture dimensions of 120 µm by 82 µm deep (Panel D) and polyester mesh with aperture dimensions of 105 µm by 63 µm deep and (Panel E). Means are shown (± SEM) and mesh aperture size shown in the top right hand corner of each panel.

were assayed two and three times respectively. Thereafter, to determine the optimal conditioning period to promote feeding in devices formed from the 105 µm aperture polyester meshes, mites conditioned for 0–3 weeks (see Section 2.2) were assayed on two hens on three separate occasions for each life stage.

2.6. Statistical analyses

For comparison of feeding rates using different mesh sizes, the proportions of mites fed (proportion of the total number of mites per pouch) were compared using a binomial generalised linear mixed model (GLMM) with a logit link function and including mesh size, life stage and the interaction between the two as fixed effects. Hen identity was fitted as a random effect. Mite feeding and mortality rates after different periods of mite starvation were compared using binomial generalised linear models (GLMs) with a logit link function and including starvation period, life stage and the interaction between the two as explanatory factors. Post hoc pair-wise comparisons between starvation periods at each life stage were conducted from the GLM estimates, with the corresponding p-values adjusted to control for false

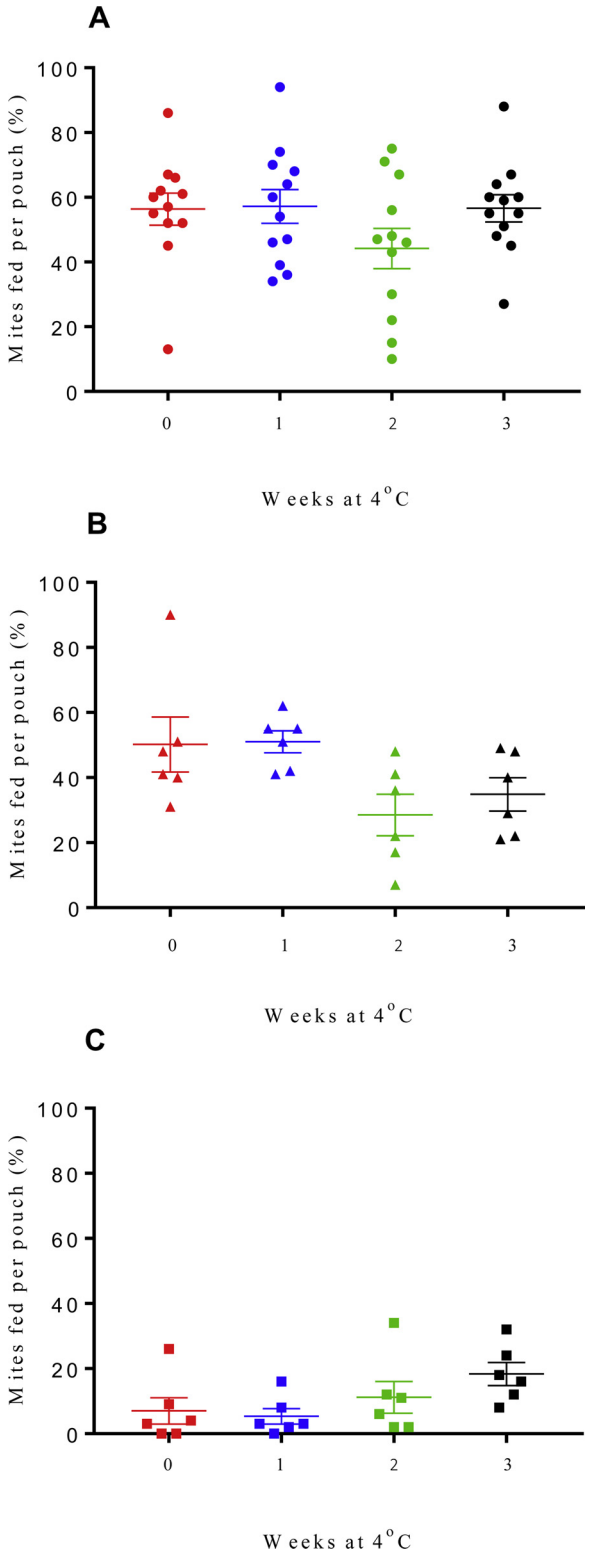

Fig. 3. Feeding success of hematophagous life stages of *Dermanyssus gallinae* feeding on hens through a polyester mesh with aperture dimensions of 105 µm wide by 63 µm deep after different starvation protocols ("Weeks at 4 °C"). Panel A shows data for adult mites; Panel B deutonymphs and Panel C protonymphs. Means are shown (± SEM).

discovery rate (FDR). A 5% significance level threshold was used for concluding statistical significance in all cases. The statistical analyses were conducted on the R system for statistical computing v3.4 (R Core Team, 2018).

3. Results

For the two nylon meshes with a greater pore depth (aperture widths of 75 µm and 125 µm) mean feeding rates (as a percentage of total mites recovered) for adult mites were 38% (range 0–80%) and 30% (range 0–55%) respectively (Fig. 2A and B). For deutonymphs, the mean feeding rates using these meshes were lower at 4% and 7%; and, for protonymphs, even lower, 2.5% and 1% respectively (Fig. 2A and B). Feeding rates for the 68 µm aperture width polyester mesh were highly variable for adults with a mean feeding rate of 62% (range 14–100%) and for deutonymphs 16% (range 3–33%) and very low for protonymphs (mean 0.5%, range 0–2%) (Fig. 2C). For this reason, the remainder of the study focussed on the meshes with the larger aperture widths and shallower depths: The 120 µm nylon mesh gave mean percentage feeding rates of 51% (range 19–100%), 19% (range 4–30%) and 5% (range 0–10%) for adults, deutonymphs and protonymphs respectively (Fig. 2D) whereas the 105 µm polyester mesh gave a mean percentage feeding rate of 59% (range 7–95%) for adults, 26% (range 4–59%) for deutonymphs and 10% for protonymphs (range 0–33%) (Fig. 2E) and was the best performing mesh with the least variability in mite feeding overall for the three life stages. Statistical analysis demonstrated that, for adults and deutonymphs, there was no statistically significant difference in the mean feeding rates between meshes with aperture widths of 105 and 120 µm ($p = 0.20$ and $p = 0.65$ respectively), but protonymphs had a statistically significantly higher feeding rates through the mesh with an aperture width of 105µm than that with an aperture of 120 µm ($p = 0.02$), probably because of the shallower aperture depth of the former. Protonymphs and deutonymphs fed at reduced rates relative to the adults through either mesh (odds ratios 0.25 and 0.35 respectively; $p < 0.01$). Therefore, the 105µm mesh was selected and used in subsequent studies to examine the effects of mite conditioning on feeding rates. A significant reduction in the feeding rate of adults was demonstrated when they had been starved for 2 weeks at 4 °C, compared with other conditioning periods (Fig. 3A). Mean deutonymph feeding rates were statistically significantly higher if the mites had been starved for 0 or 1 week at 4 °C (Fig. 3B), ($p < 0.01$) compared with 2 and 3 weeks starvation. Protonymph feeding rate (Fig. 3C) was highest after 3 weeks starvation at 4 °C, with an 18% mean feeding rate and feeding rate was significantly higher with 2 and 3 weeks starvation than with either 0 or 1 week ($p < 0.01$). Following feeding, adult mite mortality was statistically significantly lower in mites conditioned for 2 and 3 weeks at 4 °C compared to those without a conditioning period ($p < 0.01$ in both cases). No post-feeding statistically significant differences in mortality rates of protonymphs (no mortality observed) or deutonymphs ($p = 0.13$) conditioned for the various times were observed.

4. Discussion

Recently, our group has been involved in the assessment of prototype vaccines to control *D. gallinae* and we have developed and routinely used *in vitro* feeding devices to feed blood and antibodies from immunised hens to small numbers of mites to identify effective vaccine antigens (Bartley et al., 2015; Wright et al., 2016) before moving into field trials which use 750–800 hens in each trial and can last up to 6 months (Bartley et al., 2017). This strategy, while useful, has several major drawbacks:

- Data from the *in vitro* feeding devices are highly variable because inconsistent numbers of mites feed in each individual feeding device, possibly because the device lacks some of the natural feeding cues for the mites (cf. Thomas et al., 2018).
- There is a high background mortality of mites when using the *in vitro* system which may be caused by the high temperatures required to induce feeding and/or the addition of anticoagulants to the blood meal (McDevitt et al., 2006).

- Failures of the membranes through which the mites feed leads to loss of technical replicates.
- Our previous studies have shown that vaccine efficacy measured using the *in vitro* feeding device is not always translated into mite population reduction in the field trials (Bartley et al., 2015, 2017). This is a major issue because it means that vaccines that appear promising in the *in vitro* system are then tested using hundreds of birds in the field trials where they may fail to live up to their initial promise.
- The field trials involve large numbers of birds continually exposed to parasites for prolonged periods, with the attendant welfare and health issues associated with such infestations - a rise in corticosterone and adrenalin levels in infested hens is indicative of the stress associated with infestation with this parasite (Kowalski and Sokół, 2009).

Here, by optimising the depth and width of a mesh aperture size to allow poultry red mites, of all haematophagous stages (body sizes of unfed protonymphs 400 × 240 µm; deutonymphs 590 × 330 µm: adult female 920 × 520 µm (Sikes and Chamberlain, 1954), to access host skin to feed whilst still containing the mites and preventing their escape, we have produced a device which will lead to both reduction and refinement in the use of animals in research. This optimised system will allow researchers to pre-screen new vaccines and systemic acaricides/insecticides which have been developed for the control of these blood-feeding parasites before progressing to large field trials or to perform vector-capacity assessments in highly controlled conditions.

For optimal use, the device should be constructed from 105 µm aperture width, 63 µm depth, polyester mesh and the mites (irrespective of life stage) should be conditioned with no access to food for 3 weeks at 4 °C for optimal feeding and post-feeding survival.

Conflict of interest

The authors have no conflict of interest to declare.

Ethics approval and consent to participate

All experimental procedures described here were approved by the Moredun Research Institute Experiments and Ethics Committee (approval E67/17) and were conducted under the legislation of UK Home Office Project License (reference P46F495BD) in accordance with the Animals (Scientific Procedures) Act of 1986. The experimental design incorporated NC3Rs principles and adhere to the ARRIVE guidelines.

Acknowledgements

We gratefully acknowledge funding from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), project reference NC/R001081/1. We thank the Bioservices Group at Moredun Research Institute for their ongoing help and expertise and are grateful to Prof. Lise Roy (Montpellier University) for her helpful advice on *D. gallinae* morphology.

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