

Effects of *in ovo* vaccination with herpes virus of turkeys against Marek's disease on chicken performance in the absence of a challenge with Marek's disease virus

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Summary

This study tested the effects of *in ovo* vaccination with or without herpes virus of turkeys (HVT) on the performance of broiler chickens in the absence of a challenge with Marek's Disease virus. It also investigated how the concentration of HVT, determined using real-time quantitative PCR, varied with vaccine dose and age of the bird. Female Cobb 500 broiler chickens ($n = 320$) were either not vaccinated, vaccinated *in ovo* on day 18 of embryonation with 4000 or 8000 pfu of HVT, or injected with diluent alone (sham-vaccination). Measurements were made of liveweight, feed intake, feed conversion ratio, and weights of breast muscle, small intestine, abdominal fat pad, liver, bursa of Fabricius, thymus and spleen. Administration of HVT *in ovo* at the highest dose reduced liveweight and feed intake relative to the sham-vaccinated treatment and had no major effects on feed conversion ratio or organ weight. The process of *in ovo* injection alone improved feed conversion at week 7. These results suggest that the process of *in ovo* injection is beneficial to performance in the absence of a challenge, but administration of HVT vaccine during *in ovo* vaccination neutralises this effect. The concentration of HVT recovered from spleen tissue of HVT-vaccinated birds increased over time, but the dose of vaccine had no effect.

Keywords: broiler performance, *in ovo* vaccination, herpes virus of turkey, Marek's disease

Introduction

Poultry meat has an estimated annual value of \$1.3 billion in Australia, and 676,000 tonnes of chicken meat are produced per year (Austats 2004). Poultry production has changed substantially in the past fifty years in terms of efficiency and the scale of production. The time required to produce a market-ready broiler in developed countries decreased from 10 weeks in the 1950s to 6–7 weeks in 2005. The efficiency of feed utilisation has also improved (Aho 2001), and

costs associated with feed, labour and maintenance have decreased.

Improved production has been mediated through improvements in genetic selection, vaccination, temperature control, lighting schedules, stocking densities and nutrition. However, vaccine development is probably the single most important factor. The implementation of effective vaccination schedules has enabled the scale of production to evolve from small, extensive enterprises consisting of 50–200 birds to intensive production systems consisting of flocks of up to two million birds (Cook 2000, 2002).

Currently, chickens are vaccinated against infectious bursal disease, Newcastle disease, infectious bronchitis and Marek's disease (MD) (Lacy 2001). The control of MD, a ubiquitous lymphoproliferative disease, is particularly important for the poultry industry. Although vaccination is unable to produce sterile immunity, it protects birds from the immunosuppressive effects of the disease, tumour formation and death (Powell and Payne 1993; Islam *et al.* 2002). Vaccination of broilers has improved performance and vitality, and decreased the incidence of condemned carcasses (Payne 1985).

Vaccines against MD comprise two non-pathogenic strains of Marek's disease virus (MDV) or attenuated pathogenic strains, administered singly or in combination. The most commonly used and most economical vaccine is the herpes virus of turkeys (HVT), a non-pathogenic strain of Marek's disease virus (Witter 1985). In the past, HVT vaccine was administered to chicks on the day of hatch by intramuscular or subcutaneous injection (Powell and Payne 1993). However, automated *in ovo* vaccination on day 18 of embryonation (Sharma and Burmester 1982; Gildersleeve *et al.* 1993) is now the main route of vaccination of broiler chickens against MD. Improvements in feed conversion efficiency without adverse effects on hatchability or liveweight have been reported to occur after *in ovo* vaccination with HVT (Gildersleeve *et al.* 1993).

Vaccination with HVT against MD relies on stimulation of an immune response to produce long-term protection from the disease. Although HVT-vaccination has been shown to improve broiler performance in the presence of a challenge with MDV, reports of the effects of vaccination in environments that are not contaminated with MDV are equivocal. Vaccination, or an equivalent stimulation of the immune response, has been reported to affect the partitioning of energy into muscle or fat reserves (Henken and Brandsma 1982), reduce liveweight, feed intake and impair feed conversion efficiency (Klasing *et al.* 1987; Chamblee *et al.* 1992) or to have no effect on feed intake or liveweight (Henken and Brandsma 1982; Islam *et al.* 2001). However, Islam *et al.* (2001) reported an improvement in feed conversion efficiency in MDV-free broilers after vaccination with HVT, and the effect increased as the dose of vaccine increased. A positive effect of vaccination in addition to its protective effect against disease would help offset the cost of vaccination against MD, currently estimated at approximately four cents per bird. The aim of this study was to differentiate between the effect of *in ovo* injection per se and that of the vaccine.

Materials and methods

The experiment was a complete randomised design with four treatments and 12 replicates. We used 320 female commercial Cobb 500 broiler chickens (Baiada Poultry Pty Ltd., Tamworth, Australia), which were hatched from a 42-week-old parent flock. The parent birds were vaccinated against MD with Rispens CVI988 vaccine so that the chicks would contain maternal antibody to serotype-1 MDV. The experimental treatments ($n = 80$) were: HVT 4000, vaccinated *in ovo* on day 18 of incubation with 4000 plaque forming units (pfu) of HVT; HVT 8000, vaccinated *in ovo* on day 18 of incubation with 8000 pfu of HVT; SHAM, injected *in ovo* on day 18 of incubation with diluent only; UNVACC, no treatment on day 18 of incubation.

Birds were vaccinated at the hatchery (Baiada Poultry Pty. Ltd., Kootingal, Australia) using the automated INOVOJECT® method (Embrex Inc. NC, USA). The vaccine was a live cell-associated HVT (Strain FC 126, Batch no. 3203; The Marek's Company, North Ringwood, Vic. Australia). Doses were administered using 100 μ l of the diluent supplied by the manufacturer. All birds, including those of the unvaccinated treatment, were vaccinated against infectious bronchitis (IB) at hatch using aerosol and a live IB virus of the Vic S strain (Fort Dodge, Vic, Australia).

The experiment was conducted in four climate-controlled rooms at the University of New England, Armidale, Australia. The rooms, hallway and cages were cleaned, disinfected and treated with a virucide (Virkon S, Antec International Janos Hoey Pty. Ltd., Forbes, NSW Australia) prior to the commencement of the

experiment. Rooms were maintained under positive pressure, and temperature and humidity were controlled using a computerised system.

Chicks were brooded from days 0–14 in 24 multi-tiered mini-brooders with 0.406 m² cage floor space (0.03–0.04 m²/bird). On arrival, birds were permanently marked using toe-web cut marks and allocated to treatments. Each treatment was divided into groups of 11 or 12 birds and weighed. Each group of chicks was randomly allocated to one of 24 mini-brooders (six replicates per treatment). From days 14–56 of the experiment, chickens were reared in four rooms in 48 group AME cages (12 cages per room) with a cage floor space of 0.322 m² (0.06–0.07 m²/bird). All rooms contained the same number of chickens from each treatment. On day 7, birds were individually identified with wing-tags.

Feed and water were provided in galvanized iron troughs and were available *ad libitum*. Birds were fed a commercial broiler starter crumble diet from day 0–17 and a commercial broiler finisher diet from day 18–49 (Ridley Agriproducts Pty. Ltd., Tamworth, Australia). The birds were also provided with water-soluble multi-vitamins on day 36 (CCD Animal Health, Girraween, Australia) because of mortality due to ascites.

Temperature at the start of the brooding period was set to oscillate between 34°C and 38°C (average 36°C). Temperature was reduced in increments of 2°C every two days until a temperature of 22°C was reached on day 14. From day 14, the temperature of all rooms was set to oscillate between 21°C and 23°C until termination of the experiment on day 49. Data loggers, which measured temperature and humidity hourly, were placed in the rooms on day 35. The lighting schedule consisted of continuous light for the first five days and a 12-hour light-dark cycle from day 5.

Liveweight was recorded on a brooder group basis on day 0. Birds were weighed individually on days 7, 14, 21, 28, 35, 42 and 49. Feed intake was calculated on a weekly basis for each pen of birds on days 7, 14, 21, 28, 35, 42 and 49. Chickens were killed at various stages of the experiment to collect and weigh organs (Table 1). All organs were weighed immediately after dissection and the small intestine weight included that of digesta.

Spleen samples collected for viral quantification were rinsed with sterile phosphate-buffered saline (PBS), transferred to sterile eppendorf tubes containing 20 μ l of RNA Later® (Qiagen Pty. Ltd., Clifton Hill, Vic, Australia), and stored at –80°C. DNA was extracted from spleen samples (10 mg) using QIAamp DNA Mini Kits (Qiagen Pty Ltd, Clifton Hill, Vic, Australia) and the DNA content of each sample was determined spectrophotometrically. MDV and HVT viral copy number per ng of extracted DNA was determined using a qPCR method (Islam *et al.* 2004; Islam *et al.* 2005a). Absolute quantification of MDV was based on plasmid standard curves of known concentration of the target sequence for each virus. Viral load per 10⁶ spleen cells was determined assuming 2.5 pg genomic DNA per cell (Gregory, 2005) and equal extraction efficiency for

cellular and viral DNA. The number and source of samples analysed for HVT and MDV viral load by qPCR assay is shown in Table 2.

Organ weights were analysed as a percentage of liveweight. HVT data were log transformed [$\text{Log}_{10}(y+1)$] prior to analysis. Appropriate general linear models for the effects of treatment, room and their interaction were fitted using SuperANOVA® (Abacus Concepts Inc., CA, USA). As bird numbers in each treatment/room combination varied, least square means were corrected for an imbalanced design. The experimental unit for liveweight (day 7–49), organ weights, HVT load and MDV load was the individual chicken. The experimental unit for liveweight on day 0 was the brooder group, and for FCR and feed intake, the grow-out pen (3–5 birds). Repeated measures analysis was used for liveweight, feed intake and FCR. Repeated measures analysis was followed by analysis within each time period to test the effects of treatment, room and their interaction. Duncan's new multiple range test was used to identify significant differences between individual treatments in instances in which significant main effects of treatment occurred. Specific linear contrasts developed *a priori* were used within the univariate model. A probability level of $P \leq 0.05$ was assumed to be significant.

Results

No MDV was detected in spleens collected on days 35 ($n = 8$) or 49 ($n = 20$) from unvaccinated or sham-vaccinated birds. Spleen samples from unvaccinated and sham-vaccinated birds were negative

for HVT. The proportion of HVT-positive chickens was not significantly affected by HVT dose (26/31 and 23/31 for HVT 4000 and 8000 pfu, respectively; $P = 0.54$). Analysis of the HVT content of the spleens of chickens positive for HVT revealed that there was no significant effect of dose of vaccine ($P = 0.9$). However, there was a significant effect of the day of collection ($P < 0.0003$): HVT content of spleens increased from day 0–14 and plateaued on days 35–49. Back-transformed means for HVT content were 186, 535, 1142, 3150 and 3409 virus copies per 10^6 spleen cells on days 0, 7, 14, 35 and 49, respectively.

Mortality during the experiment was 6%. There was no significant effect of room ($P = 0.317$), HVT dose ($P = 0.919$) or injection on day 18 of incubation ($P = 0.468$). Post mortem examination revealed that broiler ascites was the cause of death in 60% of cases.

Analysis of liveweight data on day 0 revealed a significant effect of treatment ($P < 0.001$). This was due to the significantly higher weight of sham-vaccinated birds (43.2 ± 0.42 g) than HVT-vaccinated birds (40.2 ± 0.29 g and 40.4 ± 0.31 g for 4000 and 8000 pfu treatments, respectively). Unvaccinated birds (41.7 ± 0.17 g) were significantly heavier than HVT4000 birds but lighter than SHAM birds.

Repeated measures analysis of liveweight data from day 7 onwards revealed an effect of vaccination treatment ($P < 0.02$) and day ($P < 0.0001$), and an interaction between these two effects ($P < 0.02$) (Figure 1). There was no significant effect of room ($P = 0.42$), or interaction between room and treatment ($P = 0.38$), but there was significant interaction between the effects of room and day ($P < 0.0001$).

Table 1 Summary of sample collection.

Day	Birds per treatment	Total no. of birds	Organs weighed	Spleens stored per treatment	Total no. of spleens
0	10	40	Spleen, liver, bursa	5	20
7	10	40	Spleen, liver, bursa, thymus	5	20
14	10	40	Spleen, liver, bursa, thymus	5	20
35	4–6	20	None	4–6	20
49	39–41	161	Spleen, liver, bursa, abdominal fat pad, breast muscle, small intestine	10	40

Table 2 Number of spleen samples collected for determination of HVT and MDV viral load using qPCR assay.

Day of collection	Treatment							
	SHAM		UNVAC		HVT 4000		HVT 8000	
	HVT	MDV	HVT	MDV	HVT	MDV	HVT	MDV
0	–	–	–	–	5	–	5	–
7	–	–	–	–	5	–	5	–
14	–	–	–	–	5	–	5	–
35	4	4	4	4	6	–	6	–
49	10	10	10	10	10	–	6	–
Total	14	14	14	14	31	–	31	–

Analysis within sampling times revealed significant effects of treatment to day 35 ($P < 0.05$) and effects which approached significance on days 42 ($P = 0.066$) and 49 ($P = 0.093$). This effect was due to a reduction in liveweight of birds vaccinated with 8000 pfu of HVT on all days compared to sham-vaccinated and unvaccinated chickens. The effect of vaccination with 4000 pfu of HVT was intermediate on days 21, 35, 42 and 49.

Contrasts between HVT-vaccinated and sham-vaccinated chickens were significant ($P < 0.05$) up to day 28 and approached significance between days 35 and 49 ($P < 0.1$). HVT-vaccinated birds were lighter in each case. Reduced liveweight was greatest when chickens were vaccinated with 8000 pfu of HVT (Figure 1).

Contrasts between sham-vaccinated and unvaccinated chickens were not significant. Final body weights on day 49 did not differ between sham-vaccinated and unvaccinated birds (2961 ± 34 g and 2960 ± 35 g, respectively).

Repeated measures analysis of cumulative feed intake to week 7 revealed no significant effects of treatment, room or interaction between them. Within-week analysis revealed significant differences ($P < 0.05$) between treatments during weeks 6 and 7 with sham-vaccinated birds consuming significantly more feed than unvaccinated birds and those vaccinated with 8000 pfu of HVT (Figure 2). Contrasts between unvaccinated and sham-vaccinated birds approached significance during weeks 6 and 7 ($P < 0.1$) with sham-vaccinated birds consuming more feed than unvaccinated birds. Contrasts between HVT-vaccinated and sham-vaccinated birds were non-significant for all weeks ($P > 0.05$).

Analysis of cumulative FCR approached significance ($P = 0.093$) at week 7 but there was no effect of room ($P = 0.119$) or interaction ($P = 0.217$). There was no significant effect of treatment ($P = 0.169$) or interaction ($P = 0.248$) to week 6, although the effect of room approached significance ($P = 0.09$). Up to the end of week 5, there was no significant effect of

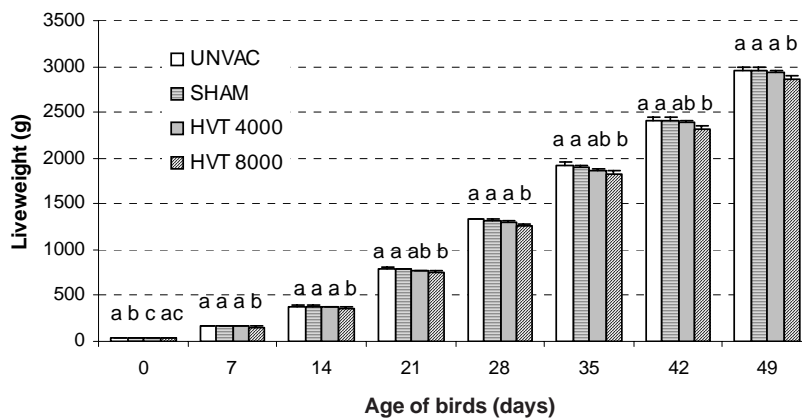


Figure 1 Least squares means (\pm SEM) for liveweight (g) of female broiler chickens at various ages either unvaccinated (UNVAC) or vaccinated *in ovo* on day 18 of embryonation with 8000 pfu of HVT (HVT 8000), 4000 pfu of HVT (HVT 4000) or diluent only (SHAM). Columns sharing a common letter are not significantly different ($P < 0.05$).

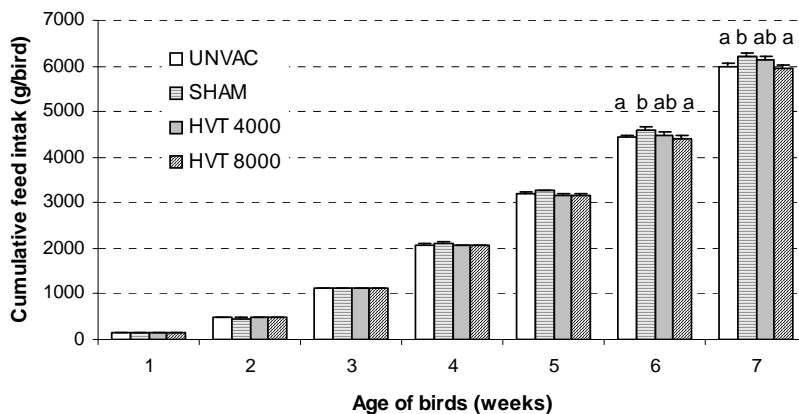


Figure 2 Least squares mean (\pm SEM) weekly feed intakes (g) of female broiler chickens to week 7 either unvaccinated (UNVAC) or vaccinated *in ovo* on day 18 of embryonation with 8000 pfu of HVT (HVT 8000), 4000 pfu of HVT vaccine (HVT 4000) or diluent only (SHAM). Columns sharing a common letter are not significantly different ($P < 0.05$).

treatment ($P = 0.495$), room ($P = 0.412$) or interaction ($P = 0.238$). Duncan's new multiple range tests revealed that cumulative FCR up to week 7 was significantly lower in sham-vaccinated chickens than in unvaccinated chickens; HVT-vaccinated chickens were intermediate between these treatments (Figure 3). Contrasts between sham-vaccinated and unvaccinated chickens for cumulative FCR were significant up to weeks 6 ($P < 0.04$) and 7 ($P < 0.02$) but were not significant up to week 5 ($P = 0.439$). This effect was manifest as a lower FCR in sham-vaccinated birds than in unvaccinated birds (Figure 3). Contrasts between HVT-vaccinated and sham-vaccinated chickens for cumulative FCR were not significant up to week 5 ($P = 0.504$), week 6 ($P = 0.224$) or week 7 ($P = 0.484$).

There were no significant main effects of treatment on weight of breast muscle ($P = 0.139$), small intestine ($P = 0.615$) or abdominal fat pad ($P = 0.942$) on day 49 (Figure 4). Significant effects of room were observed

for breast muscle weight ($P < 0.01$) and small intestine ($P < 0.04$), but there was no interaction between treatment and room ($P > 0.05$).

Contrasts between HVT-vaccinated and sham-vaccinated chickens were not significant for weight of breast muscle ($P = 0.277$), small intestine ($P = 0.494$) or abdominal fat pad ($P = 0.602$). Contrasts between Sham-vaccinated and unvaccinated birds showed that there were no significant effects of injection on relative weight of breast muscle ($P = 0.886$), small intestine ($P = 0.924$) or abdominal fat pad ($P = 0.959$).

Analysis of relative splenic mass revealed a significant effect of age of bird ($P = 0.0001$), but no significant effects of treatment ($P = 0.827$) or interaction between these two effects. Relative splenic mass increased with increasing age of the bird (Figure 5A). Contrasts between HVT-vaccinated and sham-vaccinated chickens were not significant for relative splenic mass at any age ($P > 0.05$). Contrasts between

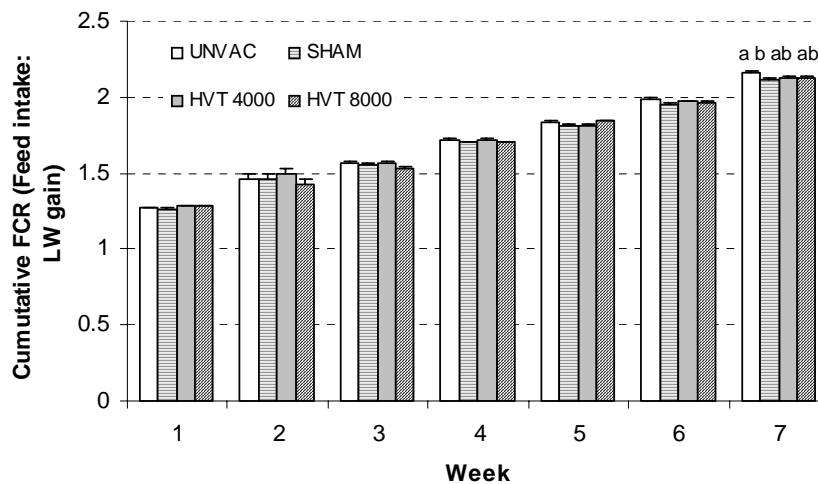


Figure 3 Least squares mean (\pm SEM) cumulative FCR of female broiler chickens either unvaccinated (UNVAC) or vaccinated *in ovo* on day 18 of incubation with either 8000 pfu HVT vaccine (HVT 8000), 4000 pfu HVT vaccine (HVT 4000) or diluent only (SHAM). Columns sharing a common letter are not significantly different ($P < 0.05$).

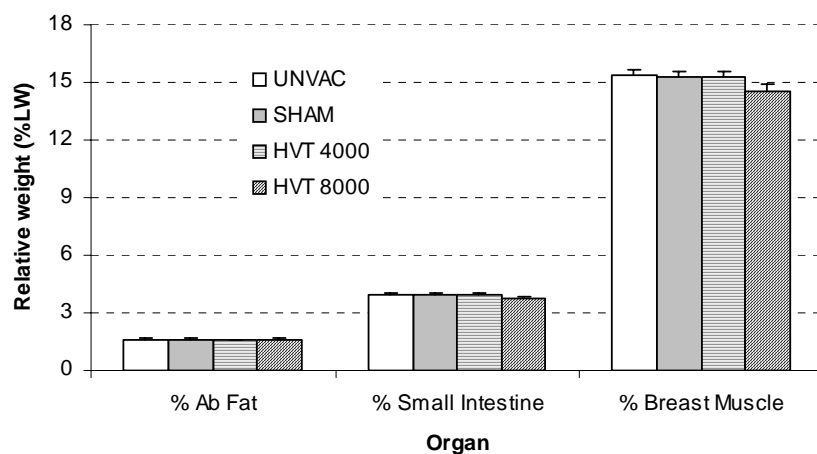


Figure 4 Relative abdominal fat pad, breast muscle and small intestine weight (LSM \pm SEM) collected from 49 day-old female broilers which were unvaccinated (UNVAC) or vaccinated with either 8000 pfu of HVT (HVT 8000), 4000 pfu of HVT (HVT 4000) or diluent only (SHAM). Treatment effects were not significantly different ($P < 0.05$).

sham-vaccinated and unvaccinated birds also revealed no significant effects of injection on splenic mass at any age ($P>0.05$).

Relative liver weight was significantly affected by age ($P<0.0001$), with no effects of treatment ($P = 0.238$). However, an interaction between these two effects approached significance ($P = 0.092$). The effect of age manifest as an increase in relative liver size to day 7 followed by a reduction to day 49, when it accounted for a smaller proportion of body weight than on day 0 (Figure 5B). A significant effect of room was found for day 49 ($P<0.002$), but there were no significant interactions between the effects of room and treatment ($P = 0.112$). Contrasts between HVT-vaccinated and sham-vaccinated chickens were not significant at any age ($P>0.05$). Contrasts between sham-vaccinated and unvaccinated chickens revealed a significant treatment effect on day 0 ($P<0.001$), with lower liver weights for unvaccinated birds than sham-vaccinated birds.

Relative bursal weight was affected by age ($P<0.0001$), but there was no significant effect of treatment ($P = 0.435$) or interaction ($P = 0.387$). The effect of age was manifest as an increase in relative weight from day 0 to day 14, followed by a reduction to day 49 (Figure 5C). Contrasts between HVT-vaccinated and sham-vaccinated chickens were not significant at any age ($P>0.05$). Contrasts between sham-vaccinated and unvaccinated chickens were non-significant at any age ($P>0.05$).

Relative thymic weight was affected by age ($P<0.001$), but there was no significant effect of treatment ($P = 0.967$) or interaction ($P = 0.409$). The

variation in relative thymic weight was manifest as a decrease in weight from day 7 to day 14 (Figure 5D). Contrasts between HVT-vaccinated and sham-vaccinated chickens were not significant at any age ($P>0.05$). Contrasts between sham-vaccinated and unvaccinated chickens were also not significant at any age ($P>0.05$).

Discussion

Vaccination with 8000 pfu of HVT reduced mean liveweights on all days up to day 49 of age. However, mean liveweight on day 49 was not significantly different between sham-vaccinated and unvaccinated broilers, indicating that the process of *in ovo* injection had no effect on this parameter.

A comparison of the liveweight of sham-vaccinated chickens with HVT-vaccinated chickens to day 49 revealed that vaccination with 8000 pfu of HVT reduced liveweight by 3.4% and vaccination with 4000 pfu of HVT reduced liveweight by 0.1%. Chamblee *et al.* (1992) reported that liveweight in broilers reared in pathogen-free environments to day 42 was reduced in broilers vaccinated with HVT compared to unvaccinated birds. A potential mechanism for this is stimulation of the immune system and a subsequent homeorhetic response (Klasing *et al.* 1987; Klasing and Johnstone 1990; Spurlock 1997). This homeorhetic response is associated with the release of the cytokines, IL-1, IL-6 and TNF, which may reduce liveweight gains by altering the partitioning of nutrients away from

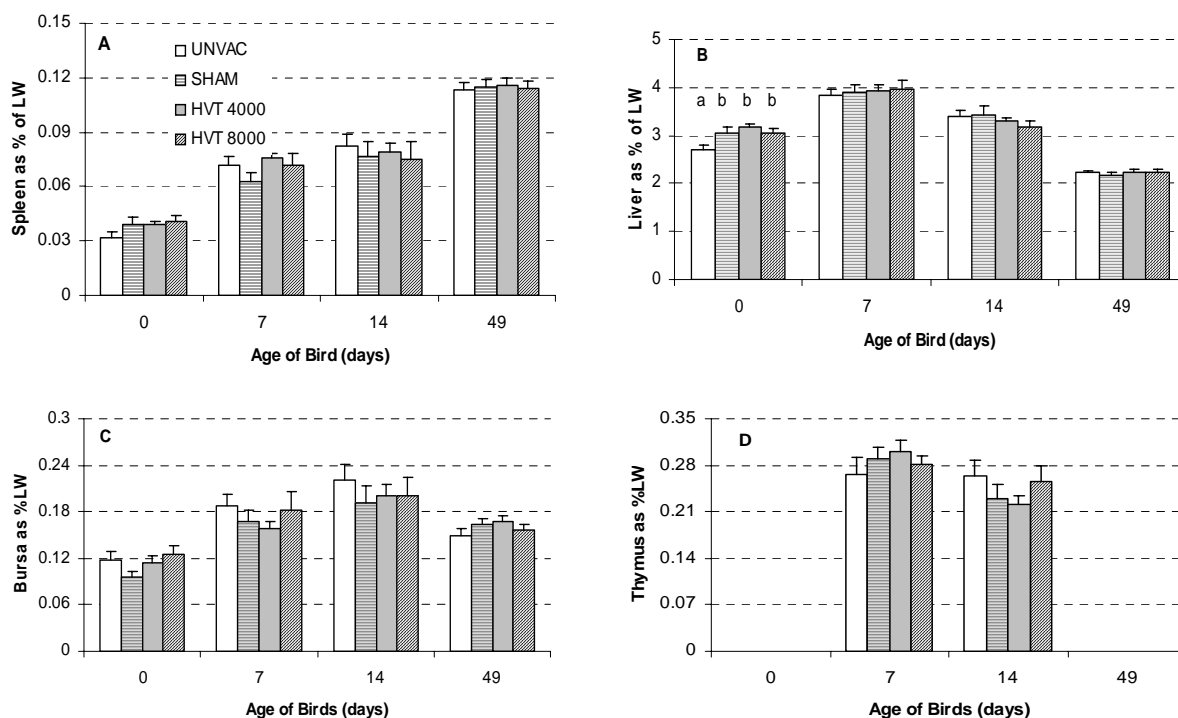


Figure 5 Least squares mean (\pm SEM) of (A) relative splenic weight, (B) relative liver weight, (C) relative bursal weight, (D) relative thymic weight collected from birds aged 0, 7, 14 and 49 days. Treatments include unvaccinated birds (UNVAC) and *in ovo* vaccination on day 18 of embryonation with 8000 pfu of HVT (HVT 8000), 4000 pfu of HVT (HVT 4000) or diluent only (SHAM). Columns without letters or sharing a common letter are not significantly different ($P<0.05$).

growth and skeletal muscle deposition towards processes that support the immune response. These cytokines also induce fever and reduce feed intake. Feed intake was reduced in HVT-vaccinated birds, suggesting that the reduction in liveweight is largely attributable to reduced feed intake. However, Islam *et al.* (2001) found no difference between the liveweights of broilers that were sham-vaccinated or vaccinated *in ovo* with 4000 pfu or 8000 pfu of HVT to 35 days of age under conditions in which birds remained free of MDV challenge.

Treatment effects on liveweight were evident as early as the day of hatch. Chicks vaccinated with HVT were lightest on day 0; sham-vaccinated chicks were the heaviest and unvaccinated birds intermediate. This suggests that *in ovo* injection can improve broiler weights at hatch, but inoculation with HVT counteracts this effect. The variation in broiler weight between treatments illustrates the ability of the immune system of the embryo to respond to antigens in the later stages of incubation and suggests that there is a metabolic cost associated with such a response as evidenced by the reduced liveweights of HVT-vaccinated birds on day 0 (Johnston *et al.* 1997). Increases in liveweight of sham-vaccinated birds on the day of hatching have not been reported previously. McCutcheon *et al.* (1982) reported that embryo weight can be increased if eggs are incubated in 60% oxygen compared to 21% oxygen, or decreased if the availability of oxygen to the egg is impaired. The availability of oxygen, particularly during the final stage of incubation, can limit embryo growth. The transfer of oxygen to the embryo requires diffusion through the shell, the shell membranes and the choriallantoic membrane (Metcalfe *et al.* 1981). The process of *in ovo* vaccination, which punctures the shell and shell membranes, may increase the availability of oxygen to the embryo by providing a direct route for transfer of air into the egg; this may explain the increased embryo weight on the day of hatching in sham-vaccinated chickens.

There were significant effects of *in ovo* injection and HVT vaccination on feed intake up to day 49. This was manifest as reduced feed intake in broilers vaccinated with the highest dose of HVT and in unvaccinated chickens, relative to sham-vaccinated birds. Reduction in feed intake has been associated with increased circulating levels of IL-1 during an immune response (Klasing *et al.* 1987). This is considered the reason for reduced feed intake after vaccination with HVT (Chamblee *et al.* 1992) or administration of antigens such as sheep red blood cells (Klasing *et al.* 1987). If HVT-vaccination reduces growth and bodyweight by altered partitioning of nutrients, reductions in feed intake associated with reduced maintenance costs may also be expected. Reductions in feed intake following immune challenge are not invariably observed. Islam *et al.* (2001) found no effect of vaccination with HVT on feed intake, and Henken and Brandsma (1982) reported that administration of sheep red blood cells did not

reduce feed intake. We have no explanation for the lower feed intake observed in the unvaccinated broilers.

Overall feed conversion ratio to the end of week 7 was lowest in sham-vaccinated broilers and highest in unvaccinated broilers; FCRs of HVT-vaccinated broilers were intermediate. This suggests that FCR can be improved through *in ovo* injection on day 18 of embryonation, but the administration of HVT vaccine reduces some of this beneficial effect.

Feed conversion ratios were significantly lower at weeks 6 and 7 in sham-vaccinated compared to unvaccinated broilers when specific contrasts were used. Feed conversion efficiency in broilers grown to 2 kg liveweight (6–7 weeks) was better after *in ovo* vaccination with HVT (2.076) than after vaccination on the day of hatch (2.095) (Gildersleeve *et al.* 1993). This was attributed to earlier provision of protection against MDV challenge. However, the results of the present study show that the effect could be partly attributable to the process of *in ovo* injection. Sham-vaccinated female broilers in the present study had a FCR of 2.12 ± 0.01 compared to 2.16 ± 0.01 for unvaccinated female broilers in the absence of challenge with MDV. The mechanisms underlying this small but significant improvement are unknown. They may include a beneficial effect of increased oxygenation as discussed previously. During the *in ovo* process, the injection needle make contact with the embryo. It is possible that this may stimulate a "wounding" response, which may facilitate improved innate immunity and thus improved protection against pathogens.

Although vaccination was improved by *in ovo* injection, there was no significant difference between the FCR of HVT-vaccinated and sham-vaccinated broilers. Islam *et al.* (2001) reported that FCR for 35-day-old broilers was improved by vaccination with HVT in a dose-responsive fashion. In contrast, Chamblee *et al.* (1992) reported impaired FCR in broilers vaccinated at hatch with HVT compared with unvaccinated control treatments. It appears that although birds vaccinated with HVT in the present study had reduced body weights, feed intake reduced correspondingly, with the result that FCR did not change. The method of vaccine administration may be also involved. In the study of Chamblee *et al.* (1992), HVT was administered at hatch, while in the case of Islam *et al.* (2001) *in ovo* vaccination was performed manually by a method shown to deposit vaccine at sites different to those at which it is deposited by the Embrex® automated *in ovo* vaccination. The study of Islam *et al.* 2001 was also performed under conditions of temperature and environmental microbiological load that would have been worse than those in the present study. It is possible that the response to vaccination may be neutral or negative for FCR under ideal conditions, in which there is a weak microbiological challenge, but beneficial under conditions of strong microbiological challenge. These beneficial effects may result from early immune system stimulation and

consequent improvement of innate and non-specific immune function, which would be useful only under conditions of challenge.

The sizes of the abdominal fat pad and small intestine were not significantly affected by vaccination with HVT or *in ovo* injection. There was a strong trend towards reduced relative breast muscle weight on day 49 in broilers vaccinated with the highest dose of HVT, reflecting the lower body weight observed for this treatment. Henken and Brandsma (1982) found that stimulation of the immune response through the administration of sheep red blood cells favoured fat deposition, but this was not supported by the present findings.

The bursa, spleen, liver and thymus were not affected by administration of HVT vaccine, indicating that HVT did not cause detectable degeneration or stimulation of these immune organs (Fabricant *et al.* 1982; Heller and Schat 1987). This is in agreement with Islam *et al.* (2002), who found that administration of 4000 pfu of HVT had little effect on bursal and thymic weights. However, it contrasts with the finding by the same authors of consistent increases in relative splenic mass on days 10–14 following vaccination with HVT subcutaneously at hatch.

The process of *in ovo* injection significantly increased relative liver weight on day 0, suggestive of a significant role for the liver during immune stimulation. The liver produces acute phase proteins in response to cytokines such as IL-1 and IL-6. It also produces some of the complement proteins and is an important antigen-trapping site, particularly of antigens arriving from the gut, as may be the case after *in ovo* vaccination into the amniotic fluid. Stimulation of the immune response has been associated with diversion of amino acids to the liver for uptake, and an increase in hepatic blood flow (Spurlock 1997).

There were significant effects of day of collection on the amount of HVT recovered from spleens of vaccinated birds, with a rapid increase in concentration from days 0 to 14, followed by a plateau at relatively high viral copy numbers on day 35. Results from the present study revealed that the amount of HVT recovered, even on day 0, and the onset of viraemia did not differ between treatments with either 4000 or 8000 pfu of HVT.

The increase in concentration of HVT recovered from spleen samples of vaccinated birds over time is consistent with the results of Sharma and Burmester (1982). In their study, the amount of HVT recovered from birds 10 days post-vaccination was greater than the amount recovered seven days post-vaccination. The results of this study are also consistent with the data of Islam *et al.* (2004) on the HVT content of the spleens of vaccinated broilers up to day 35 post-vaccination. These studies all suggest ongoing replication of HVT within the host over time, at least until day 49 of age. In contrast, Witter and Offenbecker

(1978) observed a decrease in the amount of HVT recovered from weeks 4–12. The amount of recovered HVT plateaued after 12 weeks of age, seven weeks later than in the present experiment. These variations may be attributed to the use of lower doses of HVT vaccine, layer type birds, contamination of vaccine with reticuloendotheliosis virus and the extraction methods used by Witter and Offenbecker (1978). The recent discovery that HVT is shed in dander and can be quantified in dander using qPCR has resulted in data showing that shedding of HVT peaks at day 21 post-vaccination and falls away sharply thereafter to basal levels, at least to day 60 in broiler chickens (Islam *et al.* 2005).

The amount of vaccine administered during the process of vaccination affects the amount of HVT recovered at various ages. The findings of the present study are consistent with that of Islam *et al.* (2001), who found that administration of HVT vaccine *in ovo* in doses of 4000 pfu and 8000 pfu of HVT had no effect on the timing or incidence of post vaccinal viraemia up to 35 days of age. This was attributed to vaccination at a high dosage with only a two-fold difference between doses, the same as in the present experiment. Consequently variation between these two doses in the concentration of HVT recovered from spleen samples is likely to be small, even at three days post-vaccination. The amount of HVT recovered following vaccination with very low doses of vaccine is lower than that following vaccination with very high doses. Eidson *et al.* (1974) and Witter and Offenbecker (1978) compared 100- and 1000-fold differences in HVT vaccine doses and found that less HVT was recovered from lower doses of vaccine (2 pfu and 7 pfu HVT) than from 2000 pfu, 200 pfu, and 20 pfu HVT, and 710 pfu of HVT vaccine. This effect was observed up to 3 weeks of age (Eidson *et al.* 1973) and from weeks 2–12 (Witter and Offenbecker 1978). Eidson *et al.* (1973) also found that administration of 2 pfu or 20 pfu of HVT vaccine delayed the onset of detectable vaccinal viraemia by a week in comparison to doses of 2000 pfu and 200 pfu. This is likely to be due to the low dose range and the potential interference of maternal antibodies, which is more apparent at low dose ranges.

The hypothesis that the amount of HVT recovered from spleens of vaccinated birds would increase with the age of the bird and would be affected by dose was partially supported by the results. The amount of recovered HVT from spleens increased with age but was not dose-related, even on day 0, three days after *in ovo* vaccination. The increase in the concentration of HVT over time indicates that maximum vaccinal viraemia takes several weeks to develop. Furthermore, the similarity in the concentrations of HVT recovered from the 4000 pfu and 8000 pfu HVT-vaccinated birds indicates that vaccination with the lower dose, as practiced commercially, does not compromise vaccine efficacy provided birds receive the target dose.

Conclusion

Vaccination of broilers with HVT vaccine *in ovo* reduced liveweight and feed intake. This reduction in liveweight and feed intake was greatest in birds vaccinated with the highest dose of HVT. Feed conversion efficiency, and weights of the breast muscle, small intestine, abdominal fat pad, or lymphoid organs were not affected by vaccination with HVT or the dose of vaccine.

The process of *in ovo* injection per se affected broiler performance. Feed conversion efficiency improved, and liveweight and relative liver weight at day of hatching were heavier in sham-vaccinated birds, indicating that *in ovo* injection elicits an immediate response. However, feed intake, final liveweights, and weights of organs were not affected by the process of *in ovo* vaccination.

The amount of HVT vaccine virus that can be recovered from spleens and quantified using real-time PCR was not affected by the dose of vaccine. This indicates that lower doses of vaccine (4000 pfu of HVT) can be used with no effect on vaccinal viraemia and possible benefits on liveweight gain compared to the full dose of vaccine.

Taken together, the data suggest that improved performance in response to *in ovo* vaccination with HVT is unlikely in clean environments in which there is no challenge from MDV. Similarly, there is no indication of a production penalty due to HVT-vaccination other than a slight reduction in growth, which is compensated for by reduced feed intake. The economic benefits of HVT-vaccination appear to be due to specific protection against Marek's disease virus rather than non-specific improvements in performance.

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