Response of layer and broiler strain chickens to parenteral administration of a live *Salmonella* Typhimurium vaccine

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ABSTRACT Responses to the parenteral administration of a live *aro*A deletion *Salmonella* serovar Typhimurium vaccine given to three brown egg layer strains and two broiler strains were studied. Twenty-five birds of each strain were reared together in floor pens to 6 weeks of age and then moved as individual strains to new floor pens and injected with 10^8 colony forming units (CFU) per bird of the vaccine bacteria intramuscularly or subcutaneously, 10^6 CFU per bird subcutaneously, or phosphate buffered saline (PBS) subcutaneously as a vaccination control. Three birds of one layer strain were injected intramuscularly with 0.5mg/ bird *S.* Typhimurium lipopolysaccharide (LPS) to evaluate whether response was similar for vaccine and endotoxin. Birds were weighed, and rectal temperatures recorded at the time of injection, then observed over 24 hours. Rectal temperatures were measured and blood samples collected for serum IL-6 assay at 3 hours post injection (PI). At 12 hours PI blood samples were drawn for analyses for plasma phosphorus (P), glucose (Glu), cholesterol (Cho), aspartate transaminase (AST), total protein (Ptn) and creatinine kinase (CK). Blood was sampled 14 days PI and tested for serum antibody to *S.* Typhimurium. Vaccination resulted in significant seroconversion by 14 days PI in all strains compared to the controls. The three layer strains exhibited a clinical malaise, evident within 90 minutes of injection, lasting for 12 hours, with complete recovery by 24 hours PI. Only the 10^8 CFU dose given subcutaneously produced an increase in rectal temperature 3 hours PI. Vaccination had no effect on IL-6 or Ptn. All vaccine doses increased P and the higher dose by either route decreased Cho in all bird strains. The 10^8 vaccine dose increased Glu and intramuscular injection markedly elevated CK only in the layer strains. The response was not completely congruous with that to LPS alone. The results highlight the need for consideration of differences in response of bird strain when consideration is given to the parenteral administration of live *Salmonella* vaccines.

Key words: *Salmonella*, Typhimurium, vaccine, chicken, response

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INTRODUCTION

Live *Salmonella* vaccines have been in use in the poultry industry for some time but their use has some concern from the toxicity of the endotoxins (lipopolysaccharides: LPS) contained in their cell walls especially if given parenterally (McKelvie et al., 2008). In Australia there is an interest in the use of an *aro*A deletion mutant *Salmonella* Typhimurium vaccine by parenteral injection rather than by the registered oral route (Groves et al., 2011) and work is under way to register the product for use by this route. An adverse reaction in a strain of brown egg layer chickens was previously observed following the experimental subcutaneous injection of 10^8 colony forming units of this vaccine at six weeks of age (Groves and Sharpe, 2012). When some of these birds received a second dose of the same vaccine at 12 weeks of age a proportion of them exhibited the same reaction. The reaction was thought to be possibly associated with release of endotoxin from the live bacteria following parenteral administration. This vaccine had however been used in chickens by injection at this dose rate in other chicken strains (broilers) previously without note of such a reaction (Alderton et al., 1991). Avian species are more resistant to the adverse effects of LPS than are mammals (Jones *et al.*, 1981; Fraifeld et al., 1995) and within chickens, breed differences in response to LPS from *Salmonella* have been reported (Leshchinsky and Klasing, 2001). A study was conducted to evaluate if parenteral administration of this vaccine gave a varied response in differing commercial chicken strains. Several studies have defined some physiological responses to LPS from *Enterobacteriacae* injection in chickens (Nakamura et al., 1998; Xie et al., 2000; Leshchinsky and Klasing, 2001; Baert et al., 2005; De Boever et al., 2008). Several of these reference parameters were measured in this study to examine the

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likelihood of the reaction being consistent with LPS endotoxicity.

MATERIALS AND METHODS

Animal Ethics

Experimental procedures involving animals used in this study were approved by the Birling Animal Ethics Committee (Approval Number 1038/12/11AU). All procedures were carried out in accordance with the *Animal Research Act of NSW* (1985) and Regulations (2005) following the National Health and Medical Research Council of Australia (NHMRC) guidelines (2008) and the NHMRC/Australian Research Council Code of Conduct (2007).

Experimental Facility

A poultry house was fitted out with 32 floor pens each of 6.5m^2 in area (Zootechny Pty Ltd, Austral, NSW, Australia). The pens were constructed of small gauge chicken wire sides of 1.8m height and the tops were netted to prevent bird escape. Each pen contained two plastic feed hoppers and one bell waterer (Multiquip Pty Ltd, Austral, NSW). Artificial heat for brooding was supplied from a Hired Hand^{TM} space heater with initial target temperature set at 32°C, which was reduced by 1°C every second day until 21°C was reached at 21 days of age. Photoperiod provided was 23 hours light for the first 4 days and thereafter a photoperiod: scotoperiod of 12 h:12 h was used for the remainder of the experiment. The house had an insulated roof and ventilation was provided using side curtains.

Chickens and Management

Twenty-five day old chicks of each of three brown egg layer strains (which are all Rhode Island Red by Rhode Island White hybrids), ISABROWN (**IS**), Hyline Brown (**HY**) and Hisex Brown (**HI**), and two broiler strains, Cobb 500 (**CO**) and Ross 308 (**RO**), were obtained from commercial hatcheries supplying these birds. All chicks received commercial vaccines against Marek's Disease (CVI 988), Newcastle Disease and Infectious Bronchitis at their hatchery of origin according to the hatchery's standard practices. The IS, HY and CO chicks originated from breeder flocks which had received inactivated *Salmonella* Typhimurium vaccine during their rearing whereas the HI and RO chicks did not.

On arrival at the facility, paper from the bottom of the chick delivery boxes was collected and cut into pieces, then cultured for the presence of *Salmonella* using the method described below. For each breed, five chicks were randomly selected, bled, humanely euthanized, and their internal viscera removed, pooled, cut up aseptically and cultured for detection of *Salmonella*. Culture and enrichment methods for both chick paper and viscera are described below.

The chicks were permanently identified as to their strain by making a small cut in a different toe web (outside or inside web on left or right foot) with a scalpel which had been dipped in a 1:20 dilution of an antiseptic (Dettol^{TM}: 48 mg chloroxylenol/ml; Reckitt Benckiser, West Ryde, NSW, Australia) between each bird. All experimental chicks were initially placed within the same floor pen of the experimental facility so that they could share natural exposure to any organisms present. New wood shavings was used as litter and the birds were given *ad libitum* access to a commercial bagged chick starter ration (Barastoc® Feeds), which did not contain any *Salmonella* inhibiting additives, for the first four weeks, after which they received pullet grower ration from the same supplier. At four weeks, individual numbered wing tags were applied to each bird, their toe web marking being recorded with each number. At four weeks of age the broiler strains were transferred to an adjacent pen so that their growth rate could be restricted according to broiler breeder practices (Cobb-Vantress, 2011). Half of the litter in the initial pen was moved with the broilers and fresh shavings were added to both pens to make up the floor cover. The broiler birds received 54 g of a commercial pullet grower ration per bird per day over the next two weeks. The layer strains remained in the initial pen and continued on *ad libitum* access to the same pullet grower ration.

Vaccine

Vaxsafe® ST (Bioproperties Pty Ltd, Ringwood, Victoria) is a live *aro*A deletion mutant of *Salmonella* Typhimurium developed in Australia (Alderton *et al.*, 1991). Vaxsafe® ST batch number STM071421A, with a stated label claim of 1000 bird doses at a minimum of 10^8 colony forming units (**CFU**) per dose, was used in this study. The manufacturer reported that the potency estimate for this batch was $10^{10.83}$ CFU/ml (Jackson, 2012). This was the same batch as used in a previous experiment where an adverse reaction was observed (Groves and Sharpe, 2012). One vial of the vaccine was diluted into 250 ml of sterile Phosphate Buffered Saline (PBS), such that the dilution contained at least 4×10^8 CFU per ml, based on the label claim for the batch. This dilution was used at 0.25 ml per bird to provide a dose of approximately 10^8 CFU per bird. A subsample of this solution was further diluted 1:100 in PBS to provide an inoculum of 10^6 CFU per bird when delivered in 0.25 ml. The vaccine was diluted immediately prior to inoculation and was drawn up in 0.25 ml lots in individual 1 ml sterile syringes fitted with a 1.8cm 22-gauge sterile needle. These were placed in separate plastic zip-lock bags labelled as to their vaccine concentration and kept on ice until administered. Immediately following preparation, a sample of the initial vaccine dilution (4×10^8 per ml) was submitted to Birling Avian Laboratories (Bringelly, NSW) for enumeration of *Salmonella*. The enumeration result from this was approximately 2.5×10^8 CFU/ml.

According to the manufacturer, this batch of vaccine contained approximately 9.05×10^6 endotoxin units (**EU**) per ml (Jackson, 2012). Hence, after dilution, theoretically this would have delivered approximately 3000 EU per bird at the 10^8 CFU/bird dose rate.

Salmonella Typhimurium Lipopolysaccharide

A preparation of LPS from *Salmonella enterica* serovar Typhimurium (Sigma-Aldrich, St. Louis, MO, USA), with a supplier's certificate of analysis declaring a potency of 3,000,000 EU/mg (Lot No. 089K4091), was diluted in sterile PBS to provide a concentration of 1 mg LPS per ml.

This preparation was used as a positive control endotoxin inoculation in three HY strain chicks for comparison against vaccinated HY birds at six weeks of age. The three birds were given 0.5 mg/bird intramuscularly (essentially 1.5×10^6 EU) as a comparison to the method used by De Boever et al. (2008).

Salmonella Culture and Enumeration Procedures

All microbiological testing, including the vaccine enumeration, chick paper and viscera, was performed at a NATA-accredited laboratory (Birling Avian Laboratories, Bringelly, NSW) in accordance with Australian Standard method - Microbiology of food and animal feeding stuffs: Horizontal method for the detection of *Salmonella* spp AS 5013.10–2009. This Australian method is an adaptation of the ISO 6579:2002 / Amd 1: 2007 Annex D – *Salmonella* in feces.

Briefly, the samples were initially emulsified 1:10 in buffered peptone water (BPW, Oxoid Thermo Fisher, CM509, Hampshire, UK) and incubated overnight at 37°C. Thereafter 1 ml of this enrichment was added to 9 ml of Muller-Kaufmann tetrathionate / novobiocin (MKTTn, pH 8.0 ± 0.2) broth (bioMérieux Australia Pty Ltd, Brisbane, Queensland) and incubated at 37°C overnight, and 0.1 ml of the enrichment was added to 9 ml of Rappaport-Vassiliadis Soy broth (bioMérieux, Brisbane, Queensland) and incubated at 42°C overnight. Both selective enrichments then streak-plated onto Xylose lysine deoxycholate agar (XLD) and Hektoen Enteric agar 90 mm split plates (bioMerieux Australia Pty Ltd Ref: 04175) and incubated at 37°C overnight. Isolates presumptively identified as salmonellae were confirmed using validated commercial chromogenic agar chromID™ *Salmonella* Agar (bioMérieux, Brisbane, Queensland).

Experimental Design and Procedures

At 6 weeks of age the birds were separated into their strains and randomly assigned one of four colored leg rings to allow for visual identification of the birds without having to handle or disturb them. The wing tag number of each bird assigned to a colored leg ring was recorded. The birds of each strain were placed into a new clean separate pen with fresh wood shavings as litter. They were allowed one day to accommodate to their new pen and the presence of the leg ring. Leg ring colors were randomly assigned to each vaccine treatment. Five birds with the same leg ring color were given a diluted vaccine subcutaneously at a dose rate of 10^8 CFU per bird. Another group of five was given 10^8 CFU per bird by intramuscular injection. A third group received vaccine at a dose of 10^6 CFU per bird subcutaneously and the fourth group of five birds were given a sham vaccine subcutaneously consisting of 0.25 ml of PBS.

The birds were weighed at the time of vaccination and their rectal temperatures were recorded using a digital probe thermometer (Surgipack® Digital Thermometer, ONBO Electronic Co Ltd, Shenzhen, China) accurate to ±0.1°C between 32.0 to 43.9°C. The birds were closely observed for 3 hours from outside each pen without causing disturbance.

At 3 hours post injection (PI), all birds had their rectal temperatures measured and a 1 ml blood sample was collected in serum collection tubes (Vacuette®). Serum was frozen and later assayed for chicken Interleukin-6 (IL-6) using a commercial ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, China; Catalogue No. CSB-E08549Ch). This ELISA had a stated minimum detection limit of 15.6 pg IL-6/ml.

Birds were then observed at intervals until 12 hours PI. At 12 hours PI, blood samples from all birds were collected into lithium heparin tubes (Vacuette®). Plasma from these samples were stored in aliquots and frozen. Plasma was analyzed for phosphorus (**P**), glucose (**Glu**), cholesterol (**Cho**), creatinine kinase (**CK**), aspartate transaminase (**AST**) and total protein (**Ptn**) by the University of Sydney Veterinary Clinical Pathology Unit, Camden, NSW, Australia, using Thermo Scientific reagents (details shown in Table 1) and a Konelab 20 XTi (Thermo Electron) clinical chemistry analyzer.

Statistical Analyses

Comparisons of outcomes of all factors measured were conducted using factorial ANOVA analysis, using the computer statistics package Statistica™ ver6 (Statsoft, 2003). The factors included were bird strain (5) and vaccine treatment, by dose rate and route (4). Statistical significance was accepted at $P < 0.05$.

RESULTS

Dose Rate on a Body Weight Basis

Table 2 shows initial bird body weights, and calculated dose rates of vaccine organism on a body weight basis ($\log_{10}$CFU/Kg bodyweight), as well as rectal

Table 1. Analytical methods for plasma biochemistry and enzyme studies.

Test	Method	Test kit Catalogue number[1]
Inorganic Phosphorus	Direct UV method without reduction	TR 30026
Glucose	Glucose hexakinase liquid stable reagent	TR15421
Cholesterol	Cholesterol liquid stable reagent	TR13421
Total Protein	Biuret method	TR34021/1700
Creatinine Kinase (CK)	CK activated by N-acetyl cysteine	TR14110
Aspartate Transaminase (AST)	AST (GOT) liquid stable reagent	TR70121

[1]All kits obtained from Fisher Diagnostics, Middletown, VA, USA.

Table 2. Bird starting body weights, calculated dose per kg and rectal temperatures at 0 and 3 hours post injection.

Bird type	Strain	Vaccine dose rate and and route	Mean Body weight at start (gm)	Vaccine dose Log_{10}(CFU/Kg bodyweight)[3]	Mean Rectal Temperature at hours PI (°C)		Change in mean rectal temperature 0–3 hrs PI (°C)
					0 hrs	3 hrs	
Layer	IS	Sham s/c[1]	542		41.7	41.7	$0.0^{a,b,c}$
		10^6 s/c[1]	504	6.29	41.7	41.8	$0.1^{a,b,c}$
		10^8 s/c[1]	560	8.25	41.7	42.5	0.9^{a}
		10^8 i/m^2	577	8.24	41.9	41.8	$-0.1^{b,c}$
	HY	Sham s/c[1]	541		41.8	41.7	$-0.1^{b,c}$
		10^6 s/c[1]	595	6.23	41.7	41.7	$0.0^{a,b,c}$
		10^8 s/c[1]	540	8.27	41.8	42.0	$0.2^{a,b,c}$
		10^8 i/m^2	620	8.21	41.8	42.0	$0.2^{a,b,c}$
	HI	Sham s/c[1]	444		42.0	41.9	$-0.1^{b,c}$
		10^6 s/c[1]	426	6.38	42.1	42.1	$0.0^{a,b}$
		10^8 s/c[1]	441	8.36	42.1	42.2	$0.2^{a,b}$
		10^8 i/m^2	424	8.37	41.8	42.0	$0.2^{a,b}$
Meat	CO	Sham s/c[1]	2219		41.0	41.4	$0.5^{a,b}$
		10^6 s/c[1]	1987	5.70	41.6	41.4	$-0.1^{b,c}$
		10^8 s/c[1]	2116	7.68	41.5	42.1	$0.6^{a,b}$
		10^8 i/m^2	2125	7.68	41.4	41.3	$-0.1^{b,c}$
	RO	Sham s/c[1]	2105		41.6	41.2	-0.4^{c}
		10^6 s/c[1]	2207	5.66	41.4	41.3	$-0.1^{b,c}$
		10^8 s/c[1]	2237	7.65	41.2	41.9	$0.7^{a,b}$
		10^8 i/m^2	2211	7.65	41.2	41.2	$-0.1^{b,c}$
		P =	*0.63*		*0.051*	*0.48*	*0.03*
Main Effects							
Layer			518^{b}		41.8^{A}	42.0^{A}	0.1
Meat			2148^{a}		41.4^{B}	41.5^{B}	0.1
		P =	*<0.01*		*<0.001*	*<0.001*	*0.92*
	IS		546^{B}		41.8^{A}	41.9^{A}	0.2
	HY		574^{B}		41.8^{A}	41.9^{A}	0.1
	HI		434^{B}		42.0^{A}	42.1^{A}	0.1
	CO		2190^{A}		41.4^{B}	41.6^{B}	0.2
	RO		2112^{A}		41.4^{B}	41.4^{B}	0.0
		P =	*<0.001*		*<0.001*	*<0.001*	*0.45*
		Sham s/c[1]	1170		41.6	41.6^{B}	0.0^{B}
		10^6 s/c[1]	1144		41.7	41.7^{B}	0.0^{B}
		10^8 s/c[1]	1179		41.6	42.2^{A}	0.5^{A}
		10^8 i/m^2	1192		41.6	41.7^{B}	0.0^{B}
		P =	*0.65*		*0.84*	*<0.001*	*<0.001*

[1]CFU/bird by subcutaneous injection.

[2]CFU/bird by intramuscular injection.

[3]calculated mean dose received per bird on a kg body weight basis.

Means within a column with different superscripts differ ($^{a,b,c}P < 0.05$; $^{A,B}P < 0.01$).

temperature measurements at time of injection and 3 hours PI. The broiler strains were significantly heavier than the layer strains but there were no significant weight differences within strains at time of injection. The heavier broilers obviously received a lower dose per kg of the vaccine organism at the same dose rates, however the layer strains receiving 10^6 CFU/bird did receive a lower dose rate per kg bodyweight (Log_{10} 6.23–6.29 CFU/kg) than the broilers which received 10^8 CFU/bird (Log_{10} 7.65–7.68 CFU/kg).

Clinical Observations

After 90 minutes PI, all of the layer strain birds (IS, HY and HI) which received injected vaccine exhibited a malaise typified by somnolence, depression and huddling. The HY birds which received 0.5 mg LPS showed a similar malaise but also had marked diarrhoea at this time. Diarrhoea was not observed in any other group. The controls appeared active and behaved normally. No birds of the broiler strains (CO, RO) exhibited any observable clinical effect from the injections.

Three hours PI the layer strains which received 10^8 CFU by either route were still huddled and sleepy. Some birds of the layer strains that received 10^6 CFU subcutaneously showed some activity and attempted to feed but were still noticeably less active than the controls. The birds treated with 0.5 mg LPS were also still huddled and somnolent and diarrhoea was still evident. All broiler strain birds appeared active and normal at 3 hours PI.

At 12 hours PI (at night), upon inspection, all birds were sleeping. The broiler strain birds became active when disturbed. The layer birds that had received vaccine reacted to disturbance but quickly returned to huddling and somnolent behavior. The LPS-injected birds remained huddled at 12 hours PI. By 24 hours PI, all birds appeared recovered and showed normal activity.

The broiler strains had significantly lower rectal temperatures than the layer strains (of 0.4–0.5°C) at both 0 and 3 hours PI, in agreement with the findings of Leshchinsky and Klasing (2001). After 3 hours PI only the broiler strains and IS layer strain given vaccine at 10^8 CFU/bird, and only by the subcutaneous route, showed any significant rise in rectal temperature (Table 2). HY birds given LPS intramuscularly did not show a temperature rise at 3 hours PI (Table 5).

Plasma Biochemistry and Enzymes

Plasma biochemistry and enzyme results are shown in Table 3. The 10^8 CFU/bird dose rate of vaccine by either dose route resulted in significantly elevated plasma phosphorus relative to the those given the sham dose, while those given 10^6 CFU/ bird were not significantly different to the sham dose birds. This was most evident in the RO and HI strains.

Plasma cholesterol (Cho) levels were significantly higher in the broiler strains than in the layer bird strains. There was a significant strain interaction where the 10^8 CFU/bird dose rates of vaccine by either route significantly depressed Cho compared to sham vaccinates only in the broiler strains (CO and RO) but not in the layer stains (IS, HY or HI).

There was a significant strain interaction where Glu was significantly elevated by the 10^8 CFU/bird vaccine dose given intramuscularly to the HY and subcutaneously to the HI layer strains compared with sham vaccination but this was not seen in the broiler strains nor the IS layer strain.

There were no significant differences in Ptn at 12 hours PI between strains or vaccine administration regimes.

Broiler strains of bird had significantly higher plasma AST levels than layer strains but there were no effects of administration of vaccine on AST.

The broiler breeds had significantly higher plasma CK levels in general compared to the layer strains, administration of the vaccine by the intramuscular route increased plasma CK significantly as main effect. The higher variation of CK levels within the broiler strains masked the individual variation within strains as the main effect was produced by increases in CK in the layer strains which appear non-significant within each strain.

IL-6 and ST-antibodies

IL-6 serum assays are shown in Table 4. The CO strain appeared to have lower IL-6 levels than any other strain but there was no detectable difference in IL-6 levels at 3 hours PI as a result of vaccine administration.

ELISA antibody levels to *Salmonella* Typhimurium for each treatment are shown in Table 5. Parenteral administration of the vaccine at all rates and routes resulted in significant increases in antibody titer 14 days PI compared to the sham vaccinated group. The RO strain produced a significantly higher ELISA titer than any other strain.

Comparison with LPS in HY Birds

Table 6 shows the comparisons for the HY strain only including birds treated with 0.5 mg LPS by intramuscular injection. Within this strain there were no significant differences between results from any vaccine dose or LPS for serum IL-6 at 3 hours PI, and for rectal temperature, plasma phosphorus or cholesterol at 12 hours PI. Plasma Glu and CK were elevated in birds given an intramuscular dose of vaccine at 10^8 CFU/bird as previously mentioned. However LPS administration did not change Glu or CK relative to sham or subcutaneously vaccinated treatments even though given intramuscularly in this strain. Total plasma Ptn was depressed in birds given LPS compared to the sham and 10^6 CFU/bird groups.

Plasma AST values for LPS and the higher subcutaneously administered vaccine dose may have been depressed compared to sham and other vaccine treatments ($P = 0.057$) and this may have reached significance if the number of birds in each group had been higher.

As measured by ELISA at 14 days PI, serum antibody to *Salmonella* Typhimurium in the LPS-treated HY birds was at a titer that was intermediate between the controls and the HY birds given the vaccine.

Table 3. Mean plasma biochemistry and enzymes at 12 hours Post Injection of vaccine.

Bird Type	Bird Strain	Vaccine dose rate and route	Phosphorus (mmol/L)	Cholesterol (mmol/L)	Glucose (mmol/L)	Total Protein (g/L)	AST[3] (U/L)	CK[4] (U/L)
Layer	IS	Sham	1.97	$2.32^{B,C}$	$11.83^{B,C}$	35.0	$239.8^{b,c}$	1959^{B}
		10^6 s/c[1]	1.92	$2.04^{B,C}$	11.48^{C}	37.0	$276.6^{a,b}$	2028^{B}
		10^8 s/c[1]	2.04	$1.90^{B,C}$	$12.55^{B,C}$	35.8	$245.0^{a,b}$	3429^{B}
		10^8 i/m^2	2.10	$1.94^{B,C}$	$12.88^{A,B,C}$	33.6	170.5^{c}	$9880^{A,B}$
	HY	Sham	1.72	2.50^{B}	11.38^{C}	35.8	$234.6^{b,c}$	2504^{B}
		10^6 s/c[1]	1.90	$2.30^{B,C}$	$11.68^{B,C}$	36.4	$216.0^{b,c}$	1875^{B}
		10^8 s/c[1]	2.00	$2.18^{B,C}$	$12.96^{A,B,C}$	35.2	$198.2^{b,c}$	2224^{B}
		10^8 i/m^2	2.02	$2.02^{B,C}$	15.36^{A}	35.0	$228.4^{b,c}$	$9785^{A,B}$
	HI	Sham	2.12	2.42^{B}	$12.60^{B,C}$	31.8	$243.6^{a,b}$	1528^{B}
		10^6 s/c[1]	2.00	$2.10^{B,C}$	$11.94^{B,C}$	34.2	$226.6^{a,b}$	1408^{B}
		10^8 s/c[1]	2.32	$2.00^{B,C}$	14.18^{A}	35.6	232.8^{b}	2134^{B}
		10^8 i/m^2	2.14	$1.90^{B,C}$	$13.46^{A,B}$	33.2	271.0^{b}	$10801^{A,B}$
Meat	CO	Sham	1.81	3.75^{A}	$12.63^{B,C}$	33.0	$291.3^{a,b}$	$5742^{A,B}$
		10^6 s/c[1]	2.27	$3.40^{A,B}$	$12.82^{A,B,C}$	33.4	$279.8^{a,b}$	$4971^{A,B}$
		10^8 s/c[1]	2.13	$2.93^{B,C}$	$12.08^{B,C}$	34.5	$288.2^{a,b}$	$7629^{A,B}$
		10^8 i/m^2	1.97	$2.42^{B,C}$	$11.98^{B,C}$	31.2	$279.8^{a,b}$	$6905^{A,B}$
	RO	Sham	1.95	4.38^{A}	11.48^{C}	38.5	$280.5^{a,b}$	$4321^{A,B}$
		10^6 s/c[1]	2.06	4.14^{A}	11.60^{C}	35.4	329.6^{a}	14165^{A}
		10^8 s/c[1]	2.44	$2.78^{B,C}$	11.40^{C}	31.0	$263.8^{a,b,c}$	$5231^{A,B}$
		10^8 i/m^2	2.23	$2.95^{B,C}$	$12.26^{B,C}$	33.0	$249.5^{a,b,c}$	$5812^{A,B}$
		P =	*0.13*	*<0.001*	*0.002*	*0.14*	*0.01*	*0.007*
Main Effects								
Layer			2.02	2.14^{B}	12.70^{A}	34.89	232.5^{B}	4114^{B}
Meat			2.10	3.34^{A}	12.03^{B}	33.84	283.8^{A}	6922^{A}
		P =	*0.12*	*<0.001*	*0.008*	*0.12*	*<0.001*	*0.004*
	IS		$2.01^{A,B}$	2.05^{B}	$12.19^{A,B}$	35.3	233.0^{B}	4324
	HY		1.91^{B}	2.25^{B}	12.85^{A}	35.6	219.3^{B}	4097
	HI		2.15^{A}	2.11^{B}	13.05^{A}	33.7	243.5^{B}	3968
	CO		$2.04^{A,B}$	3.12^{A}	$12.38^{A,B}$	33.0	284.8^{A}	6311
	RO		2.17^{A}	3.56^{A}	11.69^{B}	34.5	280.8^{A}	7382
		P =	*0.004*	*<0.001*	*<0.001*	*0.06*	*<0.001*	*0.08*
		Sham	1.91^{B}	3.07^{A}	11.98^{B}	34.8	258.0	3211^{B}
		10^6 s/c[1]	$2.03^{A,B}$	2.80^{A}	11.90^{B}	35.3	265.7	4889^{B}
		10^8 s/c[1]	2.19^{A}	2.36^{B}	$12.63^{A,B}$	34.4	245.6	4129^{B}
		10^8 i/m^2	2.09^{A}	2.25^{B}	13.19^{A}	33.2	239.8	8637^{A}
		P =	*<0.001*	*<0.001*	*<0.001*	*0.11*	*0.12*	*<0.001*

[1]CFU/bird by subcutaneous injection.

[2]CFU/bird by intramuscular injection.

[3]Aspartate Transaminase.

[4]Creatinine Kinase.

Means within a column with different superscripts differ ($^{a,b,c}P < 0.05$; $^{A,B,C}P < 0.01$).

DISCUSSION

There were clearly different clinical and physiological reactions to parenteral administration of the live *aro*A deletion *Salmonella* Typhimurium vaccine between the broiler and layer bird types. This should alert producers to be aware of possible breed differences in response when similar vaccines are used by this route.

Some of the observed responses are at variance with previously published studies using LPS in chickens. Within the HY strain, there were notable significant differences in response from the administration of vaccine and LPS, which were dependent on dose and route of delivery. Other studies have used a variety of LPS types (*E. coli* or *Salmonella*), administered by different routes (often intravenous), and in different chicken strains of differing ages, which may explain many of the variations observed (Adler and DaMassa, 1978; Jones et al., 1981; Baert et al., 2005). Many factors have been implicated in the febrile response to LPS in chickens including ambient temperature, age, route of administration and breed (De Boever et al., 2008). Leshchinsky and Klasing (2001) described a more marked response in body temperature following an injection of LPS in a layer strain compared to a broiler strain and hypothesized that selection for enhanced early growth characteristics also selected against the ability to mount an inflammatory response. In contrast, Cheng et al. (2004) demonstrated a varying degree of hypothermia induced by LPS administration within lines of the same layer

Table 4. Mean Interleukin-6 in serum 3 hours PI.

Bird Type	Strain	Vaccine dose rate and route	Serum IL-6 3hr PI Log_{10} (pg/ml)
Layer	IS	Sham PBS s/c[1]	2.38[a,b]
		10^8 s/c[1]	2.36[a,b]
		10^8 i/m^2	2.18[a,b]
	HY	Sham PBS s/c[1]	2.35[a,b]
		10^8 s/c[1]	1.86[a,b]
		10^8 i/m^2	1.80[a,b]
	HI	Sham PBS s/c[1]	1.74[a,b]
		10^8 s/c[1]	2.37[a,b]
		10^8 i/m^2	2.47[a]
Meat	CO	Sham PBS s/c[1]	2.00[a,b]
		10^8 s/c[1]	2.02[a,b]
		10^8 i/m^2	1.28[b]
	RO	Sham PBS s/c[1]	2.36[a,b]
		10^8 s/c[1]	2.40[a,b]
		10^8 i/m^2	2.00[a,b]
		P =	*0.04*
Main Effects			
Layer			2.16
Meat			2.00
		P =	*0.23*
	IS		2.31[a]
	HY		2.01[a]
	HI		2.19[a]
	CO		1.77[b]
	RO		2.25[a]
		P =	*0.03*
		Sham PBS s/c[1]	2.17
		10^8 s/c[1]	2.20
		10^8 i/m^2	1.95
		P =	*0.13*

[a,b]Means within a section without common superscripts differ significantly ($P < 0.05$).

[1]CFU/bird by subcutaneous injection.

[2]CFU/bird by intramuscular injection.

Table 5. Serum *S.* Typhimurium antibody ELISA titers 14 days PI.

Bird Type	Strain	Vaccine dose rate and route	ST serum antibody[3] Log10 (ELISA titre) n = 5
Layer	IS	Sham PBS s/c[1]	1.32
		10^6 s/c[1]	3.29
		10^8 s/c[1]	3.02
		10^8 i/m^2	3.35
	HY	Sham PBS s/c[1]	0.69
		10^6 s/c[1]	2.76
		10^8 s/c[1]	2.25
		10^8 i/m^2	2.79
	HI	Sham PBS s/c[1]	0.92
		10^6 s/c[1]	3.24
		10^8 s/c[1]	2.94
		10^8 i/m^2	2.94
Meat	CO	Sham PBS s/c[1]	0.00
		10^6 s/c[1]	3.11
		10^8 s/c[1]	3.36
		10^8 i/m^2	2.56
	RO	Sham PBS s/c[1]	2.26
		10^6 s/c[1]	3.58
		10^8 s/c[1]	3.56
		10^8 i/m^2	3.18
		P =	*0.13*
Main Effects			
Layer			2.32
Meat			2.59
		P =	*0.13*
	IS		2.74[B]
	HY		2.13[B]
	HI		2.51[B]
	CO		2.26[B]
	RO		3.14[A]
		P =	*<0.001*
		Sham PBS s/c[1]	1.04[B]
		10^6 s/c[1]	3.19[A]
		10^8 s/c[1]	3.03[A]
		10^8 i/m^2	2.96[A]
		P =	*<0.001*

[1]CFU/bird by subcutaneous injection.

[2]CFU/bird by intramuscular injection.

[3]S. Typhimurium antibody x-OvO ELISA mean titer.

[A,B]Means within a column with different superscripts differ ($P < 0.01$).

strain. So responses to LPS across the literature have shown marked variation in chickens.

Jones et al. (1981) reported no effect of *E. coli* LPS administered intravenously on plasma P in broiler chickens and our results are consistent here.

Xie et al. (2000) reported a hypoglycemia at 12 hours PI of *S.* Typhimurium LPS in broiler strain chickens, hypothesized to be due to an increased insulin secretion from LPS-induced endotoxemia. In comparison, in the current study, an increase in plasma Glu was observed at the same time mark following administration of live attenuated *S.* Typhimurium vaccine by the intramuscular route at the highest dose rate, but only in the layer strains. Elevation in blood glucose may involve responses to a corticosterone release due to a stress reaction to the physical effect of a harmful incident (Fudge, 2000), in this case possibly the result of an irritating intramuscular injection. Muscle irritation or inflammation appears to be the predominant effect on blood glucose levels as the same dose delivered subcutaneously caused no change. Glucocorticoid response to a stress can be immediate and will result in hepatic gluconeogenesis proportional to the severity of the stress (Greco and Stabenfeldt, 1997). Chickens tolerate endotoxin much more than mammals, the latter often experiencing a fatal hypoglycemia upon significant exposure (Jones et al., 1981) and this observed difference may be partially responsible for this tolerance.

An increase in serum CK, without an increase in liver enzyme activities, such as AST, is a specific indicator of muscle damage (Fudge, 2000). In birds it is known that trauma, such as an irritant injection, can markedly elevate serum CK, as can vigorous muscular activity associated with rough handling or surgically invasive events (Fudge, 2000). This increase in CK was observed

Table 6. Comparison of vaccine and ST LPS injection in HY layers.

Vaccine Treatment	n	Mean serum IL-6 at 3 hrs PI log10(pg/ml)	Mean Rectal temperature change 0–3 hrs PI (°C)	Mean Plasma P (mmol/L) 12 hrs PI	Mean Plasma Cholesterol (mmol/L) 12 hrs PI	Mean Plasma Glucose (g/L) 12 hrs PI	Mean Plasma Total protein (g/L) 12 hrs PI	Mean Plasma AST (U/L) 12 hrs PI	Mean Plasma CK (U/L) 12 hrs PI	ST[4] antibody 14 days PI (mean Log_{10} ELISA titer)
Sham	4	2.35	−0.1	1.72	2.50	11.4^{B}	35.8^{a}	235	2504^{B}	0.69^{b}
10^6 s/c[1]	3	2.32	0.0	1.90	2.30	11.7^{B}	36.4^{a}	216	1875^{B}	2.76^{a}
10^8 s/c[1]	4	1.86	0.2	2.00	2.18	13.0^{B}	$35.2^{a,b}$	198	2224^{B}	$2.25^{a,b}$
10^8 i/m^2	5	1.80	0.2	2.02	2.02	15.4^{A}	$35.0^{a,b}$	228	9785^{A}	2.79^{a}
LPS[3] 0.5 mg i/m^2	3	2.98	0.1	2.07	2.17	11.4^{B}	27.7^{b}	180	2227^{B}	$1.61^{a,b}$
SEM[5]		0.14	0.07	0.05	0.07	0.36	0.80	9.48	631	0.23
P =		*0.075*	*0.61*	*0.41*	*0.29*	*<0.001*	*0.04*	*0.057*	*<0.001*	*0.01*

[1]CFU/bird by subcutaneous injection.

[2]CFU/bird by intramuscular injection.

[3]*Salmonella* Typhimurium lipopolysaccharide.

[4]*S.* Typhimurium antibody x-OvO ELISA mean titer.

[5]Standard error of the mean.

Means within a column with different superscripts differ ($^{a,b}P < 0.05$; $^{A,B}P < 0.01$).

only in the layer strains in this experiment. Muscle damage alone does not explain the malaise as subcutaneous administration of the vaccine also produced the clinical effect in layers and injection of a high dose of *S.* Typhimurium LPS intramuscularly in a layer strain caused malaise but did not elicit a CK response.

Blood P levels in birds can fluctuate widely but an elevated level is unusual, perhaps seen frequently only in association with renal failure (Fudge, 2000). Xie et al. (2000) reported increased plasma P following LPS administration and suggested that this could be due to reduced P excretion, in turn due to transient impairment of kidney function related to an acute phase reaction. However there were no other indications of renal impairment or damage in birds given vaccine in this experiment; there was no observed diarrhoea which would have resulted from a diuresis if the vaccine had elicited kidney damage. LPS administration to the HY strain did not result in a change in blood P whereas diarrhoea was prominent.

In the present study, plasma Cho levels decreased 12 hours PI in birds given the vaccine at 10^8 CFU by either route. This was most apparent in the broiler strains and there was some indication of this being dose-dependent. There were numerical reductions in Cho in the layer strains and the response in the HY strain was similar to that elicited with injection of LPS (results approached significance in a small number of birds). This is consistent with earlier findings in Cobb broilers following intravenous inoculation of 5 mg/kg Typhimurium LPS (Xie et al., 2000). Infection and inflammation and LPS injection in mammals, including humans, consistently result in significant hypercholesterolemia (Feingold et al., 1993). This increase in serum cholesterol is accomplished by impairment of the reverse cholesterol transport (RCT) system (de Beer et al., 2013). The RCT process involves removal of cholesterol from the body, predominantly through macrophages (Majdalawieh and Ro, 2009) and subsequent efflux of free cholesterol to the liver where it is excreted in the bile. The second step in the RCT pathway is suppressed by the activity of LPS (Majdalawieh and Ro, 2009). Cholesterol metabolism is complex and shows marked species variation even among mammals. Kushner (1982) described a hypocholesterolemia as a result of moderate tissue damage in humans. The results of the present study are in contrast with most of the literature on effects of LPS administration.

Xie et al. (2000) observed an elevation in IL-6 in broilers 12 hours after intravenous injection of 5 mg/kg of Typhimurium LPS in broiler chickens (CO strain). In contrast in the present study the administration of the vaccine intramuscularly caused a decrease in plasma IL-6 in this strain of bird. Differences here could also relate to the different LPS levels and route of administration.

There were some differences in response of the HY strain between vaccine and LPS administration. Theoretically at least, the amount of endotoxin within the live vaccine would be very low compared to an intentional LPS administration. Although bird numbers compared here were low, this may indicate that the malaise induced by the vaccine in layer strains may not be due solely to an endotoxic effect from LPS. The difference in serum antibody response of this strain to the vaccine and to a large dose of LPS is a further indication that the bird is reacting, at least serologically, to far more than just the LPS contained within it.

Overall, the observed physiological responses of the birds were in some respects similar to but not completely consistent with the effects of intramuscular injection of *S.* Typhimurium LPS. As LPS is widely used as a model for inflammation in a variety of animals, researchers using this technique in chickens need to be aware of possible variations due to bird strain. The vaccine used in this study is currently undergoing assessment for use by a parenteral route. Producers need to be aware that there may be a transient reaction to this vaccine given by this route in some breeds of chicken.

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