ABSTRACT As part of a program to study the pathological effects of coccidia infections on growth, we have examined the relationship of plasma L-arginine (ARG) levels to infective doses of *Eimeria acervulina* and infection-associated changes in weight gain, plasma carotenoids, and plasma NO$_2^{-}$ + NO$_3^{-}$. Chickens consuming a starter ration containing 1.68% ARG were infected with a range of doses of *E. acervulina*. At 6 d postinoculation (PI), weight gains were significantly reduced by infections with 5×10$^5$ and 1×10$^6$ oocysts per chick (OPC). Gross lesion scores of chickens infected with 5×10$^4$ through 1×10$^6$ OPC were significantly greater than scores of chicks infected with 1×10$^3$ OPC. Compared with levels from uninfected controls, plasma NO$_2^{-}$ + NO$_3^{-}$ concentrations were significantly increased by infection with 5×10$^5$ (Key words: coccidiosis, nitric oxide, inflammation, weight gain, amino acids)

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INTRODUCTION

L-arginine (ARG) is an essential amino acid for chickens (Tamir and Ratner, 1963; Boorman and Lewis, 1971) and must be supplied in the diet in balanced proportions to other amino acids for maximum growth (Griminger and Scanes, 1986). Anorexia and nutrient malabsorption that are characteristic of coccidia infections (Long, 1968; Ruff and Allen, 1990) can decrease the availability of ARG and other amino acids. Available ARG could also be decreased through its utilization as a substrate for NO synthesis, which has been found to accompany infection with several coccidia species (Allen and Teasdale, 1994; Allen 1997a,b). The impact of this latter process on the body ARG pool is not known. It is important, therefore, to determine whether production of NO as an inflammatory response to coccidiosis can significantly reduce circulating concentrations of ARG and thus affect growth. As part of a larger study on how coccidia infections affect muscle metabolism (Fetterer and Allen, 2000), we report data describing the effects of increasing infective doses of *Eimeria acervulina* on weight gain, plasma ARG, and carotenoids as well as effects on NO$_2^{-}$ + NO$_3^{-}$, an in vivo index of NO production caused by up-regulation of induced nitric oxide synthase (iNOS) (Stuehr and Marletta, 1985; Nussler et al., 1994; Prada and Kremsner, 1995).

MATERIALS AND METHODS

**Chickens, Housing, and Diet**

Male, SexSal chicks (White Rock × Rhode Island Red) were obtained at 1 d of age and raised in Brower brooders to 3 wk of age, at which time they were transferred to suspended wire cages, five chicks per cage. They were maintained at temperatures between 25 and 28 C and were provided fresh water and broiler starter ration ad libitum. This ration had a guaranteed analysis of at least 24% crude protein, 4% fat, and 4% crude fiber and was

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Moyer’s Hatchery, Quakertown, PA 18951.
Brower, Houghton, IA 52631.
Southern States Broiler Starter Ration 4050, Southern States Cooperative, Upper Marlboro, MD 20772.

**Abbreviation Key:** ARG = L-arginine; iNOS = induced nitric oxide synthase; OPC = oocysts per chick; PI = postinoculation.
analyzed to contain 1.64% ARG, 0.88% methionine + cysteine, and 24.0% protein, providing 3,200 kcal ME. During the period of infection, uninfected and infected chickens were housed under separate but equivalent conditions to prevent accidental infection of the controls.

**Parasite**

_Eimeria acervulina_ is a laboratory strain purified by single oocyst isolation from an Alabama field strain and propagated at this laboratory by periodic passage through chickens. This species parasitizes the duodenal mucosal epithelium of the chicken primarily. Oocysts, isolated from feces and cleaned were stored in 2.5% K$_2$Cr$_2$O$_7$ at 4 C. For use, the K$_2$Cr$_2$O$_7$ was washed out.

**Experimental Protocol**

Fifty chicks were randomized into five groups of 10 chicks each with statistically equivalent (P > 0.05) mean weights (Gardiner and Wehr, 1950). Each group was given one of the following doses of _E. acervulina_ oocysts per chick (OPC): 0, 5 × 10$^{3}$, 5 × 10$^{4}$, 5 × 10$^{5}$, and 1 × 10$^{6}$.

At 6 d post inoculation (PI) chickens were weighed, bled, killed, and scored for gross duodenal lesions (Johnson and Reid, 1972).

**Blood Samples and Analyses**

Blood samples were collected by cardiac puncture into EDTA-treated tubes$^{5}$ and centrifuged at 2,200 × g for 10 min at 4 C; the resulting plasma was frozen at −20 C in 1-mL aliquots until analyzed.

**Carotenoids.** Plasma carotenoids were extracted with acetone and analyzed spectrophotometrically as described previously (Allen et al., 1996). Results were expressed as µg lutein equivalents/ml plasma.

NO$_3^−$ + NO$_2^−$. Plasma samples were filtered through Centricon 30$^{6}$ spin filters to obtain clear, colorless filtrates. These were diluted 1:3 with distilled water, and total NO$_2^−$ and NO$_3^−$ in 150 µL of the diluates were analyzed colorimetrically in a 96-well microtiter plate format using the Griess reaction after reducing + NO$_3^−$ to NO$_2^−$ with nitrate reductase according to the method of Verdon et al. (1995). The results were expressed as µM concentrations in the plasma filtrates.

**Arginine.** Plasma samples (500 µL) were precipitated with 25 µL of 70% perchloric acid and centrifuged at 12,000 × g for 10 min at 4 C; the resulting supernatants were retained for arginine analysis. Arginine was measured using HPLC following precolumn derivatization with o-phenaldialdehyde using a modification of methods used for plasma amino acids (Fekkes et al., 1995). Fifty µL perchloric acid supernatant was diluted with 345 µL water, neutralized with 100 µL 0.3M LiOH, and 5 µL of 2.5 µmol/mL norvaline$^{7}$ was added as the internal standard. Sequentially, 5 µL o-phenaldialdehyde reagent$^{8}$ and 5 µL sample or standard was drawn into the autosampler (Waters auto-sampler model 7177) needle and allowed to react for 1 min. Five µL of the mix were injected onto the column. Elution was performed with a multisegment gradient, and fluorescence was monitored at an excitation wavelength of 337 nm and an emitting wavelength of 452 nm with a Waters$^{9}$ model 474 detector. The standard consisted of an amino acid solution$^{9}$ with norvaline in 0.1 N HCl at a final concentration of 25 nmol/mL. Arginine concentrations were quantitated by an internal standard method using Waters 7$^{th}$ Millenium 32 analytical software. The elution time of arginine was confirmed by addition of a known amount of arginine to plasma samples. Recovery of arginine was greater than 90%. Results were determined as nmol arginine per ml plasma and reported as µM.

**Statistics**

Statistical analyses were performed using the General Linear Models, Duncan’s Multiple Range, and NPARIWAY procedures of SAS (SAS, Inc., 1990).

**RESULTS**

At 6 d PI, bird weight gains were significantly reduced by infections with 5 × 10$^{3}$ and 1 × 10$^{6}$ OPC as compared with untreated controls (Table 1). Gross lesion scores of chickens infected with 5 × 10$^{4}$ through 1 × 10$^{6}$ OPC were significantly greater than scores of chicks infected with 1 × 10$^{5}$ OPC. Compared with concentrations from uninfected controls, plasma NO$_3^−$ + NO$_2^−$ concentrations were significantly increased by infection with 5 × 10$^{5}$ and 1 × 10$^{6}$ OPC, plasma concentrations of ARG were significantly decreased by infection with 5 × 10$^{4}$ through 1 × 10$^{6}$ OPC, and plasma carotenoids were significantly decreased by all infection doses. Among the groups of infected chicks, plasma arginine was significantly correlated with plasma carotenoids ($r^2 = 0.98128; P > 0.0187$) but not with infective dose or weight gain; plasma NO$_2^−$ + NO$_3^−$ was positively correlated ($r^2 = 0.99575; P > 0.0043$) with dose and negatively correlated ($r^2 = −0.98421; P > 0.0158$) with weight gain (Table 1). Regression analysis of the measured variables indicated that the strongest relationship existed between plasma ARG and carotenoids. A best fit equation was determined to be: $y = 101.94 ln x + 168.65$ ($R^2 = 0.9741$) where $y =$ plasma ARG and $x =$ plasma carotenoids. The parallel decreases in plasma ARG and carotenoids with increasing oocyst dose are shown in Figure 1.

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$^{5}$Monovette, 9 mL, potassium EDTA anticoagulant; Sarstedt, Inc., Newton, NC 28658-0468.

$^{6}$Amicon Inc., Beverly, MA 01915.

$^{7}$Sigma Chemical, St. Louis, MO 63178.

$^{8}$Waters, Inc., Milford, MA 01757.

$^{9}$Standard H, Pierce Chemical Co., Rockford, IL 61105.
amino acid to NO during an inducible nitric oxide synthase (iNOS), conversion of this ARG is the only known precursor for NO synthesized by infected with Eimeria acervulina, accompanied by reduction of plasma ARG that may be caused by malabsorption and not related to utilization of ARG through increased iNOS activity.

In conclusion, the results of this experiment indicate that reduced weight gain during E. acervulina infection might also reduce the metabolic availability of ARG and negatively impact growth.

DISCUSSION

Growth of chickens requires an adequate and balanced supply of both essential and nonessential amino acids. L-Arginine is an essential amino acid and needs to be supplied through the diet because chickens lack several enzymes of the ornithine cycle and cannot biosynthesize ARG from ornithine (Tamir and Ratner, 1963; Austic, 1976; Wu et al., 1995). Some ARG synthesis can take place in the kidney and macrophages if citrulline is supplied (Grimminger and Scanes, 1986; Su and Austic, 1999), but these activities may be inconsequential in vivo. A requirement of 1.25% ARG for chicks on a diet providing 3,200 kcal ME and 23% protein has been reported (Cuca and Jensen, 1990). The National Research Council (1984) recommends 1.44%. In the present experiment, chickens consumed a diet containing an analyzed 1.64% ARG.

Plasma levels of ARG in the chick are directly influenced by diet and by catabolic processes (particularly kidney arginase) (Austic and Nesheim, 1970; Chu and Nesheim, 1979; Robbins and Baker, 1981) and in coccidia infections of the small intestine, by malabsorption associated with mucosal destruction (Ruff and Allen, 1990). It is speculated that the closer and more significant correlations of plasma NO\textsubscript{2} + NO\textsubscript{3} with dose and weight gain suggest that reduced weight gain is more directly related to the inflammatory processes associated with the immune response, such as might be elicited by IL-1 or TNF-\( \alpha \). However, the proof of this awaits further experimentation. More importantly, the data strongly indicate that, under the conditions of this experiment, malabsorption of ARG did not inhibit a significant increase in NO\textsubscript{2} + NO\textsubscript{3}, which is presumed to arise from increased iNOS activity (Stuehr and Marletta, 1985; Nussler et al., 1994; Prada and Kremsner, 1995).

In conclusion, the results of this experiment indicate that reduced weight gain during E. acervulina infection is accompanied by reduction of plasma ARG that may be predominantly caused by malabsorption and not related to utilization of ARG through increased iNOS activity.

### TABLE 1. Effects of varying doses of Eimeria acervulina on weight gain, lesion scores, plasma NO\textsubscript{2} + NO\textsubscript{3}, carotenoids, and L-arginine at 6 d postinoculation

<table>
<thead>
<tr>
<th>Oocyst dose per chick</th>
<th>Weight gain (g)</th>
<th>Lesion score</th>
<th>Plasma NO\textsubscript{2} + NO\textsubscript{3} (( \mu \text{M} ))</th>
<th>Plasma carotenoids (( \mu \text{g/mL} ))</th>
<th>Plasma L-arginine (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>112 ± 4\textsuperscript{a}</td>
<td>ND\textsuperscript{1}</td>
<td>10.65 ± 0.82\textsuperscript{b}</td>
<td>3.6 ± 0.3\textsuperscript{a}</td>
<td>288.6 ± 24.5\textsuperscript{a}</td>
</tr>
<tr>
<td>5,000</td>
<td>114 ± 3\textsuperscript{a}</td>
<td>3.3 ± 0.2\textsuperscript{b}</td>
<td>10.95 ± 0.76\textsuperscript{b}</td>
<td>2.3 ± 0.2\textsuperscript{b}</td>
<td>266.0 ± 21.4\textsuperscript{b}</td>
</tr>
<tr>
<td>50,000</td>
<td>106 ± 4\textsuperscript{a}</td>
<td>3.6 ± 0.2\textsuperscript{b}</td>
<td>12.03 ± 0.91\textsuperscript{b}</td>
<td>1.4 ± 0.2\textsuperscript{c}</td>
<td>216.6 ± 22.7\textsuperscript{b}</td>
</tr>
<tr>
<td>500,000</td>
<td>93 ± 6\textsuperscript{a}</td>
<td>3.8 ± 0.1\textsuperscript{b}</td>
<td>20.51 ± 4.47\textsuperscript{b}</td>
<td>0.9 ± 0.1\textsuperscript{d}</td>
<td>149.7 ± 9.4\textsuperscript{a}</td>
</tr>
<tr>
<td>1,000,000</td>
<td>72 ± 4\textsuperscript{a}</td>
<td>3.8 ± 0.1\textsuperscript{b}</td>
<td>27.17 ± 4.38\textsuperscript{b}</td>
<td>0.8 ± 0.2\textsuperscript{b}</td>
<td>146.1 ± 14.1\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b,c,d}Values are means ± SEM. Within columns, means with no common superscript differ significantly (\( P < 0.05 \)).

\textsuperscript{1}Not done.

**FIGURE 1.** Concentrations of plasma L-arginine and carotenoids at 6 d postinoculation as a function of oocyst dose of Eimeria acervulina.
Additionally, malabsorption of ARG does not appear to hinder a significant increase in iNOS activity. The high negative correlation between plasma NO$_2^+ +$ NO$_3^-$ and weight gain and the high positive correlation between plasma NO$_2^+ +$ NO$_3^-$ and oocyst dose suggest that reduced weight gain may be more closely tied to the inflammatory arm of the immune reaction than to coccidiosis infection.

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REFERENCES


