UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EXTRATO DE *Tagetes erecta* NA DIETA DE REPRODUTORES DE CODORNAS JAPONESAS. EFEITOS NA PRODUÇÃO, REPRODUÇÃO, INCUBAÇÃO, PERFIL OXIDATIVO E PROGÊNIE

Autora: Lidiane Staub

Orientadora: Profª. Drª. Tatiana Carlesso dos Santos

Tese apresentada, como parte das exigências para obtenção do título de DOUTOR EM ZOOTECNIA, no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá - Área de Concentração Produção Animal/Pastagens e Forragicultura

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Estado do Paraná
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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 20 de fevereiro de 2020.

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Tudo que é seu encontrará uma maneira de chegar até você.”

Chico Xavier

Ao meu pai, Ido Staub, (*in memoriam).*

E à minha mãe, Ereni Caspers Staub, por todo o amor, apoio e por tudo que me ensinaram…

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E, no dia 20 de fevereiro de 2020, submeteu-se à banca de defesa do doutorado.

ÍNDICE

Página

LISTA DE TABELAS E QUADROS xii
LISTA DE FIGURAS xiv
RESUMO xvi
ABSTRACT xviii
I. INTRODUÇÃO 20
1.Revisão Bibliográfica 22
1.1 Processo de oxidação 22
1.2 Antioxidantes 24
1.3 Os carotenoides 25
1.4 Xantofilas e o extrato de *Tagetes erecta* 27
1.5 Metabolismo dos carotenoides 28
1.6 Uso de carotenoides em dietas de aves 30
1.7 Formação do ovo, deposição de carotenoides e processos de oxidação em aves 32
1.8 Considerações finais 34
1.9 Referências 35
II. OBJETIVOS GERAIS 40
III. PERFORMANCE, EGG QUALITY, AND SERUM AND OXIDATIVE PROFILES OF JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA*) BREEDERS SUPPLEMENTED WITH *TAGETES ERECTA* EXTRACT 41
Abstract 42
Introduction 44

Material and methods 45

Results 52

Discussion 53

Conclusion 57

References 57

IV. DIETARY SUPPLEMENTATION OF JAPANESE QUAIL BREEDERS WITH *TAGETES ERECTA* FLOWER EXTRACT INFLUENCES EGG FERTILITY, INCUBATION PARAMETERS, AND PROGENY PERFORMANCE 70

Abstract 71

Introduction 73

Material and methods 74

Results 80

Discussion 82

Conclusions 87

References 88

V. CONSIDERAÇÕES FINAIS 102

LISTA DE TABELAS E QUADROS

III. PERFORMANCE, EGG QUALITY, AND SERUM AND OXIDATIVE PROFILES OF JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA*) BREEDERS SUPPLEMENTED WITH *TAGETES ERECTA* EXTRACT

Table 1 - Ingredient composition, calculated nutrient content, and energy value of the basal diet fed to Japanese quail (*Coturnix coturnix japonica*) breeders 61
Table 2 - Primers used for polymerase chain reaction amplification 622
Table 3 - Productive performance and egg quality of Japanese quail (*Coturnix coturnix japonica*) breeders fed diets supplemented with different concentrations of T*agetes erecta* extract..... 633
Table 4 - Color parameters of eggs from Japanese quail (*Coturnix coturnix japonica*) breeders fed diets supplemented with different concentrations of *Tagetes erecta* extract..... 644
Table 5 - β-Carotene and xanthophyll content in experimental diets and yolk from Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract 655
Table 6 - Serum profile of male and female Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract. 666
Table 7 - Serum antioxidant activity and lipid peroxidation in Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract..... 677

IV. DIETARY SUPPLEMENTATION OF JAPANESE QUAIL BREEDERS WITH *TAGETES ERECTA* FLOWER EXTRACT INFLUENCES EGG FERTILITY, INCUBATION PARAMETERS, AND PROGENY PERFORMANCE

Table 1 - Ingredient composition, calculated nutrient content, and energy value of basal diets fed to Japanese quail (*Coturnix coturnix japonica*) breeders and chicks at the starter and grower phase 911
Table 2 - Probability of fertility or infertility, hatchability or mortality, early, mid, and late mortality, and mortality at pipping of eggs from 12- and 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations 922

Table 3 - Weight at hatching and Pasgar score of chicks from 12- and 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 955
Table 4 - Lipid oxidation in egg yolk, yolk sac, and liver of chicks from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 999
Table 5 - Performance of starter and grower chicks from fertile eggs of 12-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 100
Table 6 - Performance of starter and grower chicks from fertile eggs of 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 1011

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

Figura 1 - Esquema geral da oxidação lipídica 23
Figura 2 - Absorção, metabolismo, e transporte de carotenoides: apo-CAR, apo-carotenoide; RAL, retinal; VLDL, lipoproteína de muita baixa densidade; LDL, lipoproteína de baixa densidade; HDL, lipoproteína de alta densidade. 29

III. PERFORMANCE, EGG QUALITY, AND SERUM AND OXIDATIVE PROFILES OF JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA*) BREEDERS SUPPLEMENTED WITH *TAGETES ERECTA* EXTRACT

Figure 1 - Superoxide dismutase (SOD) activity in the liver of Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract..... 68
Figure 2 - Relative expression of glutathione peroxidase (GPX7) and superoxide dismutase (SOD1) genes in the vaginal mucosa of Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract..... 69

IV. DIETARY SUPPLEMENTATION OF JAPANESE QUAIL BREEDERS WITH *TAGETES ERECTA* FLOWER EXTRACT INFLUENCES EGG FERTILITY, INCUBATION PARAMETERS, AND PROGENY PERFORMANCE

Figure 1 - Effect of *Tagetes erecta* flower extract concentration on the fertility and infertility probability of eggs from 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders..... 93
Figure 2 - Incubation weight loss in stored and non-stored eggs from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with *Tagetes erecta* flower extract..... 94
Figure 3 - Yolk sac weight of embryos from eggs stored (a) for 8 d before incubation or (b) non-stored eggs laid by Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 96

Figure 4 - Embryo weight as a function of egg incubation period and *Tagetes erecta* flower extract concentration in the diet of Japanese quail (*Coturnix coturnix japonica*) breeders..... 97
Figure 5 - DPPH radical scavenging activity in the yolk of fresh eggs from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 98
Figure 6 - Superoxide dismutase (SOD) activity in the liver of 1-d-old chicks hatched from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract. 98

RESUMO

No experimento I o objetivo foi avaliar os efeitos da suplementação do extrato de *Tagetes erecta* na dieta de reprodutores de codornas japonesas sobre a produção, a qualidade de ovos e as variáveis bioquímicas séricas e perfil oxidativos. Foram utilizadas 480 matrizes de codornas distribuídas em um delineamento experimental inteiramente ao acaso, com cinco dietas e doze repetições, com seis fêmeas e dois machos por unidade experimental. As dietas experimentais foram: (0; 0.1; 0.2; 0.3 e 0.4% de inclusão de extrato de *Tagetes erecta* (Tag) kg/ração). As variáveis de desempenho produtivo e qualidade de ovos não foram influenciadas (P>0.05) pelos níveis de extrato de Tag. A cor da gema foi influenciada pela inclusão do extrato de Tag, com redução linear (y = 43.6813 – 0.5116Tag) da luminosidade e efeito linear crescente para a variável a* (y = -0.6481+ 0.7922Tag). A composição de β-carotenos e xantofila nas rações experimentais e nas gemas dos ovos também apresentou valores crescentes em função da inclusão de extrato de Tag. Na análise de dienos conjugados no soro das fêmeas e de capacidade antioxidante do soro das fêmeas e dos machos não houve efeito significativo (P>0.05). Para bioquímica sérica houve efeito quadrático nos niveis de albumina (g/dL) de machos em que o maior nível de concentração (2.39 g/dL) foi estimado com inclusão de 1.87 (g/Kg) do extrato. O triglicérides (mg/dL) do soro das fêmeas foi influenciado de forma linear decrescente, em que a inclusão de 1 g de extrato de Tag, reduziu 60.92 (mg/dL) dos triglicérides no sangue. A atividade de superóxido dismutase (SOD) (U/mg) da proteina no fígado das fêmeas apresentou efeito quadrático ($y = -0.0004 + 0.0365Tag – 0.0070Tag^2$), com estimativa de maior concentração de SOD de 0.0471 (U/mg) de proteína com 2.61 g/Kg de inclusão de extrato. A expressão dos genes do sistema antioxidante SOD1 e GPX7, na mucosa da vagina de codornas reprodutoras não apresentou efeito significativo (P>0.05). No experimento II o objetivo foi avaliar os efeitos da suplementação do extrato de *Tagetes erecta* na dieta de reprodutores de codornas japonesas sobre o rendimento de incubação, a qualidade, o desempenho produtivo e a capacidade antioxidante e a peroxidação lipídica da progênie. A probabilidade do ovo ser fértil nas aves com 12 semanas de idade atingiu 100%, e não foi possível estimar o efeito da dieta. As 25 semanas, houve efeito quadrático (P=0.034) em função da inclusão dos níveis de extrato de Tag. Nas estimativas de probabilidade de eclosão, as aves com 25 semanas apresentaram maior eclosão (95.06%), em comparação com as aves com 12 semanas (90.16%). A mortalidade nas fases iniciais e médias de incubação apresentaram médias de (5.45%) e (25.93%) para aves com 12 e 25 semanas, respectivamente. Aves com 12 semanas apresentaram maior mortalidade no período final (94.55%) e com 25 semanas (74.07%). Para peso inicial ao nascimento houve efeito linear decrescente (P<0.05) em função do aumento da inclusão de Tag na dieta. A qualidade do pintinho analisada pelo escore de Pasgar© não apresentou efeito da dieta. Observou-se que os ovos que foram armazenados por até 8 dias, apresentaram perda de peso de 2.6% durante a incubação

com efeito quadrático, sem efeito da dieta. O peso do vitelo reduziu ao longo da incubação e em função da inclusão de Tag na dieta. Houve interação significativa entre a inclusão de Tag e o armazenamento ou não dos ovos. O peso do embrião apresentou efeito cúbico dos níveis de inclusão de Tag (P=0.04) e quadrático em função dos dias de incubação (P<0.0001). A capacidade antioxidante da gema não foi influenciada (P>0.05) pelos níveis de Tag. Ovos armazenados por 8 dias antes da incubação tiveram ovos e pintinhos com maiores concentrações de MDA na gema do ovo, no vitelo e no fígado, sem efeito da dieta. A atividade da SOD no fígado de codornas de 3 dias de idade não foi influenciada pelos niveis de Tag (P>0.05). Para o desempenho da progênie, as aves oriundas de matrizes com 12 semanas de idade houve efeito linear (P<0.05) (CA = 1.8941 + 0.4400 Tag) crescente para a conversão alimentar no período de 7 a 14 dias de idade, e efeito quadrático (P<0.05) para ganho de peso, com o valor de ponto máximo com inclusão estimada em 2.02 g de extrato de Tag kg/ração e conversão alimentar, com ponto de máximo de 1.67 g de inclusão de extrato de Tag kg/ração no período entre 14 e 21 dias de idade. Para as aves oriundas de matrizes com 25 semanas de idade, observou-se efeito quadrático (P<0.05) para consumo de ração e conversão alimentar no período de 7 a 14 dias de idade, com os seguintes valores de ponto máximo, 2.27 e 2.28 g de extrato de Tag kg/ração, respectivamente. Os presentes resultados mostraram que a a inclusão do extrato de Tag na dieta de reprodutores, aos níveis indicados pelo fabricante como pigmentante de gemas de ovos, não apresentam características suficientes para melhorias de desempenho produtivo, ou como potencial antioxidante do soro e dos tecidos avaliados ou em otimizar a produtividade em se tratando de incubação e desempenho da progênie, quando os animais foram criados em condições ideais de ambiente e temperatura. A estimativa de fertilidade dos ovos indicou que a inclusão do extrato de Tag desempenha papel importante na nutrição dos reprodutores, e que esta pode ser uma ferramentta importante para a melhoria desta variável. A suplementanção de extrato de Tag na dieta de reprodutores de codornas japonesas, nos níveis indicados comercialmente, apresentam boa capacidade de pigmentação, deposição de carotenoides e xantofilas nas gemas, e pode proporcionar como objetivo final pintainhos de melhor qualidade.

Palavras-chave: Carotenoides, cravo de defunto**,** reprodução, sistema antioxidante.

ABSTRACT

In experiment I the objective was to evaluate the effects of supplementation of *Tagetes erecta* extract in the diet of Japanese quail breeders on production, egg quality and serum biochemical variables and oxidative profile. 480 matrices of quails were used in a completely randomized design, with five diets and twelve replications, with six females and two males per experimental unit. The experimental diets were: (0; 0.1; 0.2; 0.3 and 0.4% inclusion of *Tagetes erecta* extract (Tag) kg / feed). The variables of productive performance and egg quality were not influenced (P> 0.05) by the levels of Tag extract. The color of the yolk was influenced by the inclusion of the Tag extract, with linear reduction (y = 43.6813 – 0.5116Tag) of luminosity and increasing linear effect for the variable a * (y = -0.6481+ 0.7922Tag). The composition of β-carotenes and xanthophyll in experimental diets and egg yolks also showed increasing values due to the inclusion of Tag extract. In the analysis of conjugated dienes in the serum of females and the antioxidant capacity of serum from females and non-males there was a significant effect (P> 0.05). For serum biochemistry, there was a quadratic effect on the levels of albumin (g / dL) in males in which the highest level of concentration (2.39 g / dL) was estimated with the inclusion of 1.87 (g / kg) of the extract. The triglycerides (mg / dL) of the female serum was influenced in a linear decreasing manner, in which the inclusion of 1 g of Tag extract reduced 60.92 (mg / dL) of the triglycerides in the blood. The superoxide dismutase (SOD) activity (U / mg) of the protein in the liver of the females showed a quadratic effect ($y = -0.0004 + 0.0365Tag - 0.0070Tag^2$), with an estimated higher SOD concentration of 0.0471 (U / mg) of protein with 2.61 g / Kg of extract inclusion. The expression of the genes of the antioxidant system SOD1 and GPX7, in the mucosa of the vagina of breeding quails did not have a significant effect (P> 0.05). In experiment II, the objective was to evaluate the effects of supplementation of *Tagetes erecta* extract in the diet of Japanese quail breeders on incubation yield, quality, productive performance and antioxidant capacity and lipid peroxidation of the progeny. The probability of the egg being fertile in birds at 12 weeks of age reached 100%, and it was not possible to estimate the effect of the diet. At 25 weeks, there was a quadratic effect (P = 0.034) due to the inclusion of Tag extract levels. In the hatching probability estimates, birds at 25 weeks showed greater hatching (95.06%), compared to birds at 12 weeks (90.16%). Mortality in the initial stages and averages of incubation showed averages of (5.45%) and (25.93%) for birds at 12 and 25 weeks, respectively. Birds at 12 weeks showed higher mortality in the final period (94.55%) and at 25 weeks (74.07%). For initial birth weight, there was a decreasing linear effect (P <0.05) due to

the increased inclusion of Tag in the diet. The quality of the chick analyzed by the Pasgar © score had no effect on the diet. It was observed that the eggs that were stored for up to 8 days, presented weight loss of 2.6% during the incubation with quadratic effect, without diet effect. The calf weight decreased during incubation and due to the inclusion of tags in the diet. There was a significant interaction between the inclusion of tags and the storage or not of eggs. The embryo weight showed a cubic effect of the levels of inclusion of Tag (P = 0.04) and quadratic as a function of the days of incubation (P <0.0001). The yolk's antioxidant capacity was not influenced (P> 0.05) by Tag levels. Eggs stored for 8 days before incubation had eggs and chicks with higher concentrations of MDA in the egg yolk, calf and liver, with no effect of the diet. The activity of SOD in the liver of 3-day-old quails was not influenced by the levels of Tag (P> 0.05). For progeny performance, birds originating from 12-week-old sows had a linear effect (P <0.05) (CA = 1.8941 + 0.4400 Tag) increasing for feed conversion in the period from 7 to 14 days age, and quadratic effect (P <0.05) for weight gain, with the maximum point value with an estimated inclusion of 2.02 g of Tag kg / feed extract and feed conversion, with a maximum point of 1.67 g of inclusion of Tag extract kg / ration in the period between 14 and 21 days of age. For birds from 25-week-old breeding stock, a quadratic effect (P <0.05) was observed for feed consumption and feed conversion in the period from 7 to 14 days of age, with the following maximum point values, 2 .27 and 2.28 g of Tag extract kg / ration, respectively. The present results showed that the inclusion of Tag extract in the breeding diet, at the levels indicated by the manufacturer as an egg yolk pigmentant, does not present sufficient characteristics for improvements in productive performance, or as an antioxidant potential of the serum and tissues evaluated or in optimize productivity when it comes to incubation and progeny performance, when the animals were raised under ideal conditions of environment and temperature. The egg fertility estimate indicated that the inclusion of Tag extract plays an important role in breeding nutrition, and that this can be an important tool for improving this variable. Supplementation of Tag extract in the diet of Japanese quail breeders, at the levels indicated commercially, has a good pigmentation capacity, deposition of carotenoids and xanthophylls in the buds, which can provide better quality chicks as a final goal.

Keywords: Antioxidant system, carotenoids, marigold, reproduction.

I. INTRODUÇÃO

A busca contínua por melhoria na alimentação de aves de produção tem levado a inclusão de extratos, ervas e especiarias na dieta, com o objetivo de substituir aditivos alimentares, reduzir a utilização de antimicrobianos, ou até mesmo para a utilização de alimentos alternativos, e seus componentes não possuem pigmentantes na composição. Exemplos são os extratos de plantas, óleos essenciais e aditivos fitogênicos. Estes produtos apresentam compostos biologicamente ativos como polifenóis, carotenoides e vitaminas, que podem atuar como antioxidantes, pigmentantes ou imunomoduladores, apresentando esses efeitos na ração, nas aves, nos ovos ou até mesmo na progênie. As substâncias antioxidantes ajudam a prevenir o estresse oxidativo por meio da remoção de intermediários de radicais livres e são, portanto, um agente efetivo contra várias doenças. Em nutrição animal, as propriedades antioxidantes são apreciadas por agir principalmente como promotores de saúde (Faehnrich et al., 2016).

A peroxidação lipídica nas aves é influenciada principalmente pela qualidade da matéria-prima, pela alta presença de ácidos graxos insaturados nos tecidos e pela ingestão inadequada de nutrientes envolvidos no sistema de defesa antioxidante. Sendo assim, a inclusão de antioxidantes na dieta das aves, visa preservar a qualidade e os níveis nutricionais do alimento e, consequentemente, proteger os tecidos da ave viva e o produto final das matrizes que são os ovos férteis e os pintos.

O processo de desenvolvimento embrionário resulta em condição altamente oxidativa e a utilização de antioxidantes na dieta de matrizes tem sido avaliada em várias espécies animais. Pesquisas têm focado nas características antioxidantes que os carotenoides apresentam e os resultados têm demonstrado que a sua presença na dieta e

transferência para o ovo pode, potencialmente, reduzir as reações de oxidação no vitelo (gema) e no organismo dos embriões em desenvolvimento oriundos desses ovos.

Baseado no fato de que o ovo está constituído por grande quantidade de substâncias lipídicas ou lipossolúveis, dispostas na gema ou vitelo, o comportamento do perfil antioxidante desse conteúdo vitelogênico durante a incubação é de fundamental importância para o desenvolvimento de tecnologias de nutrição e de incubação. Essa área de estudos representa a base fisiológica para a compreensão dos mecanismos oxidativos e suas interações durante a embriogênese e podem contribuir para o desenvolvimento de novas tecnologias.

Diferentes substâncias antioxidantes têm sido adicionadas à dieta de matrizes, ou de aves em desenvolvimento com objetivo de melhora no desempenho produtivo. Dentre estas, encontram-se o grupo dos carotenoides. As xantofilas compreendem os derivados oxigenados dos carotenos e sua ação benéfica está principalmente ligada à sua capacidade antioxidante, pela alta eficiência em absorver e estabilizar radicais livres, possuindo também grande ação como pigmentante de ovos e carnes. Dentre as xantofilas, o extrato de Marigold, ou cravo-de-defunto, é encontrado na forma de um pó amarelado, obtido principalmente da flor de *Tagetes erecta*.

A nutrição das matrizes interfere diretamente na composição dos ovos e, consequentemente, possui influência no desenvolvimento embrionário e no desempenho do pintinho pós-eclosão. Esses aspectos são comuns a todas as aves de produção. As matrizes de codornas apresentam a vantagem do baixo custo de produção e o rápido desenvolvimento, quando comparadas às matrizes de frangos de corte. Neste sentido, estudos fisiológicos dos efeitos de sustância antioxidantes no desempenho, perfil antioxidante das matrizes e os efeitos no desenvolvimento embrionário e da progênie, tornam-se bastante relevantes.

Sendo assim, o presente estudo objetivou avaliar a inclusão de níveis de extrato de *Tagetes erecta* na dieta de matrizes de codornas japonesas, sobre o desempenho produtivo, a qualidade de ovos, o desempenho de incubação e da progênie e sobre as variáveis bioquímicas séricas e antioxidantes.

1.Revisão Bibliográfica

1.1 Processo de oxidação

A oxidação é um processo extremamente importante no metabolismo dos animais, por meio da qual os nutrientes provenientes dos alimentos são oxidados com a finalidade de gerar calor, produzir energia para os processos metabólicos e transformar nutrientes em tecido corporal. Por outro lado, enquanto o oxigênio é essencial ao metabolismo, os sistemas biológicos oferecem condições favoráveis para ocorrência de reações oxidativas, que podem destruir componentes importantes dos alimentos como as vitaminas lipossolúveis (A, D, E, K) e ácidos graxos essenciais (ácidos linoleico, linolênico e araquidônico), além de danos as estruturas celulares e aos tecidos animais (Adams, 1999).

Para se tornarem estáveis, os radicais livres transferem a energia acumulada para as substâncias próximas a eles, principalmente nos ácidos graxos poli-insaturados (AGPI). Em alimentos, a formação de radicais livres (RL) ocorre pela ação direta de fontes externas de energia, como luz, calor e radiação. A oxidação dos lipídios é uma das mais importantes causas da deterioração dos alimentos, pela formação de sabores e odores indesejáveis (rancidez oxidativa), bem como a formação de substâncias tóxicas potencialmente perigosas quando ingeridas pelas aves e pelo homem (Rocha, 2011).

Os lipídios são susceptíveis ao ataque dos RL e a oxidação ocorre como reação em cadeia. Os AGPI são mais susceptíveis à oxidação, resultando na formação de alcanos, aldeídos, alcoóis e hidroperóxidos, entre outros produtos (Hogg e Kalyanaraman, 1999) (Figura 1). A maior susceptibilidade dos AGPI à oxidação se deve pela velocidade de auto-oxidação dos ácidos graxos aumenta o número de duplas ligações que eles possuem. Os AGPI com cadeias longas são particularmente susceptíveis à peroxidação (Pappas et al., 2005). A oxidação lipídica é influenciada pela temperatura, pela luz, presença de O_2, além das propriedades físico-químicas do produto e da presença de possíveis iniciadores e catalisadores da reação (Maisuthisakul et al., 2007).

A geração de RL no organismo é uma condição fisiológica contínua. Quando a produção supera a remoção, estes se acumulam causando o chamado “estresse oxidativo” que pode lesar as células, se esse acúmulo for mantido no organismo por período prolongado (Carneiro, 2013). Em condições normais, o sistema de oxidação e o

sistema antioxidante das aves domésticas estão em estado de equilíbrio dinâmico. Uma vez que o corpo produz muitas espécies reativas de oxigênio (ERO's) ou se o sistema antioxidante do corpo estiver danificado, o equilíbrio será quebrado e causará estresse oxidativo. ERO's são um dos radicais livres que podem existir independentemente e contêm um ou mais elétrons não pareados (Kannan e Jain, 2000).

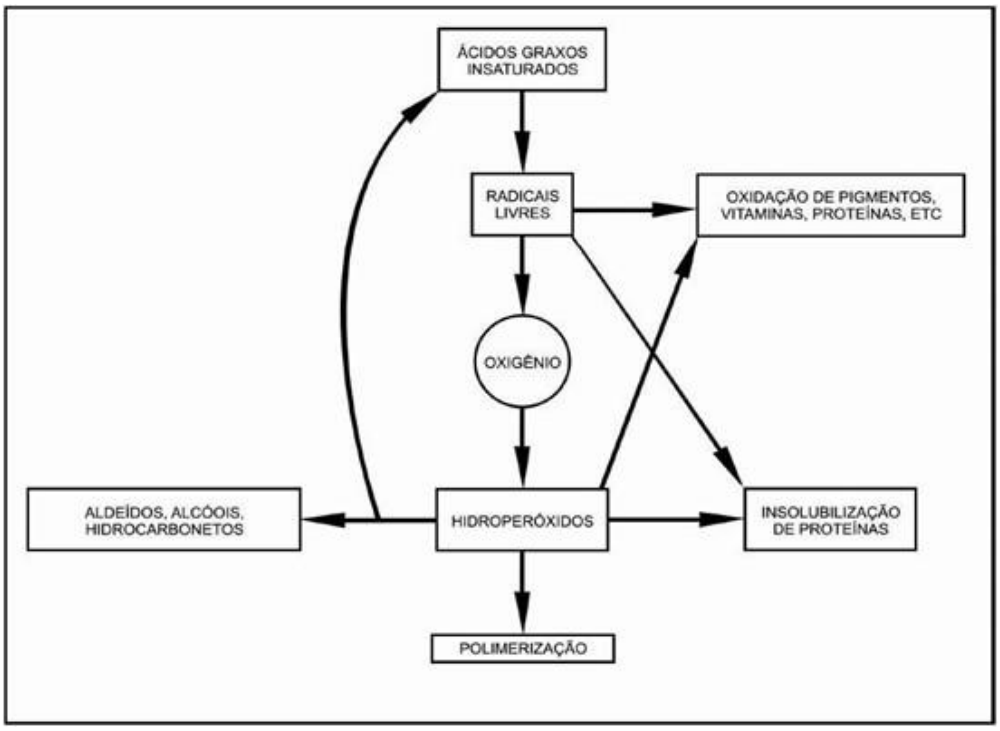

Figure 1 - Esquema geral da oxidação lipídica
Fonte: Adaptado de Jadhav et., al (1996).

De acordo com Silva et al., (1999), é possível distinguir estas três etapas de evolução oxidativa da seguinte forma:

a) desaparecimento dos substratos de oxidação – lipídio insaturado, oxigênio;

b) aparecimento dos produtos primários de oxidação – peróxidos e hidroperóxidos – cuja estrutura depende da natureza dos ácidos graxos presentes; e

c) aparecimento dos secundários de oxidação, obtidos por cisão e rearranjo dos peróxidos.

Os peróxidos são intermediários instáveis, especialmente se expostos a altas temperaturas ou em presença de metais de transição, portanto eles são decompostos pela interação com radicais livres, formando o radical peroxil (LOOH + R_ _ LOO_ + RH), ou pela ruptura da ligação oxigênio-oxigênio, que é relativamente fraca, formando os

radicais alcoxil (LO_) e hidroxil (_OH). Os produtos secundários são produzidos no decurso da decomposição dos primários. Um dos produtos secundários é o malondialdeído (MDA), que é formado durante a oxidação dos PUFA por cisão beta dos PUFA peroxidados, principalmente do ácido araquidônico (Lima e Abdalla, 2001).

Diante disso, a quantificação de malonaldeídos (MDA) formados durante a oxidação de PUFAs pode representar o status antioxidante de um tecido. A quantidade de MDA é mensurada pela concentração de substâncias reativas ao ácido tiobarbitúrico TBARs em uma determinada amostra e produz um composto de cor avermelhada sendo comumente detectada em espectrofotômetro na faixa espectral de 532, 538 e 540 nm, expressando a absorbância equivalente de MDA (mg/kg ou µ/g) (Silva et al., 1999; Rocha et al., 2010); Lee et al., 2012).

1.2 Antioxidantes

Os antioxidantes são substâncias que retardam a velocidade da oxidação por meio da inibição da produção, ou dos efeitos deletérios dos RL presentes em alimentos e nos organismos de seres vivos (Duarte-Almeida et al., 2006). São definidos como aditivos alimentares, que prolongam o tempo de conservação de alimentos, protegendo-os contra a deterioração causada pela oxidação. Eles são descritos, segundo o seu mecanismo de ação, como aditivos que reagem com RL formando produtos inativos ou quanto à sua presença em alimentos, como qualquer substância capaz de retardar ou impedir a rancidez ou outras deteriorações de sabor pela oxidação (Pokorny et al., 2001).

Alguns antioxidantes são sintetizados pelo organismo das aves. Porém, precisam de alguns precursores, como os minerais selênio, zinco, cobre, manganês e ferro, para a síntese de enzimas de função antioxidante, como a glutationa peroxidase, a tiredoxina redutase, a superóxido dismutase e a catalase. As vitaminas E, C e os carotenoides executam da mesma maneira atividade antioxidante, de forma a proteger as células da ação dos RL, reforçando a importância da presença destes nutrientes na alimentação (Surai, 2006).

Os principais antioxidantes utilizados na nutrição animal obtidos de produtos de origem vegetal, são: os compostos fenólicos, os carotenoides, a vitamina C (ácido ascórbico), a vitamina E e o selênio (Silva et al., 2010).

Para alcançar proteção eficiente, os tecidos dispõem de sistema antioxidante integrado, que consiste de um arranjo de diversos componentes lipossolúveis (vitamina E; carotenoides), hidrossolúveis (ácido ascórbico; glutatinona) e enzimáticos (glutationa peroxidase; superóxido dismutase; catalase) (McLean et al., 2005).

1.3 Os carotenoides

Carotenoides são pigmentos orgânicos sintetizados a partir de unidades de isopreno em plantas e algas. Os carotenoides representam um dos grupos mais difundidos de pigmentos naturais. Eles são sintetizados pelas plantas, mas estão presentes em animais, e se acumulam inalterados da dieta ou modificados metabolicamente (Marounek e Pebriansyah, 2018). Existem mais de 1100 carotenoides conhecidos (Yabuzaki, 2017). De acordo com Fraser e Bramley (2004), essas substâncias podem ser encontradas com cores entre o amarelo e o vermelho.

A estrutura dos carotenoides é baseada em um esqueleto de isoprenoide C40 que pode ser acíclico ou ter uma ou ambas as extremidades modificadas em aneis. Os carotenoides de hidrocarbonetos são classificados como carotenos e aqueles que contêm pelo menos um átomo de oxigênio são as xantofilas (Yonekura e Nagao, 2007).

Nutricionalmente os carotenoides são classificados em:

- carotenoides pró-vitamínicos, representando os que têm atividade de pró-vitamina A, ou;
- carotenoides inativos, que desempenham apenas atividade antioxidante ou corante (Olson, 1999).

Já quimicamente, podem ser divididos em dois grupos:

- carotenoides hidrocarbonados, denominados carotenos; e
- carotenoides oxigenados, denominados xantofilas (Goodwin, 1965).

Estes dois grupos principais podem ser estruturalmente subdivididos em sete grupos, com seus principais representantes:

1. Hidrocarbonetos – carotenos (α, β, γ e δ) e pelo licopeno;
2. Álcoois – xantofilas (criptoxantina, zeaxantina e luteína);
3. Cetonas – equinenona, cantaxantina e astacina;
4. Epóxidos – flavoxantina;
5. Éteres – espiriloxantina;
6. Ácidos – crocetina;

7. Ésteres – bixina e crocina;

Os carotenoides são aditivos não alimentares que podem ser adicionados à dieta. Além de serem pigmentantes, possuem atividades antioxidantes, protegendo as células contra os processos de oxidação. São conhecidos como precursores de Vitamina A, e possuem importante papel antioxidante, pigmentantes de pró-Vitamina E imunomoduladoras, pois removem RL, absorvem e dissipam o excesso de energia destes e reciclam a Vitamina E (Williams et al., 1998).

Os carotenoides constituem um dos mais importantes grupos de pigmentos naturais pela larga distribuição, diversidade estrutural e inúmeras funções. São responsáveis pelas cores laranja, amarela e vermelha das frutas, hortaliças, flores, algas, bactérias, fungos, leveduras e animais, que apesar de não sintetizarem tais moléculas, podem obtê-las a partir do consumo de alimentos de origem vegetal (Ribeiro e Seravalli, 2004).

A proteção antioxidante é fornecida pelos carotenoides acíclicos, que possuem nove ou mais duplas ligações conjugadas. Eles são capazes de retirar do meio espécies altamente reativas de oxigênio (MCbride, 1996). A ordem crescente de capacidade de sequestrar o oxigênio por parte dos carotenos e xantofilas é: licopeno, astaxantina ou cantaxantina, β-caroteno, luteína e crocina (Fontana et al., 2000).

Os carotenoides são eficientes na interação com oxigênio e são igualmente eficazes para neutralizar os RL. Os carotenoides, ao combaterem as espécies reativas de oxigênio, podem interagir de três maneiras diferentes: transferência de elétrons; remoção de íons de hidrogênio ou adição de espécies radicalares (Morais, 2006). No entanto, a atividade antioxidante é altamente dependente de vários fatores, como por exemplo, o tipo de carotenoide e sua ação com o meio.

As aves não sintetizam carotenoides, portanto, estes devem ser obrigatoriamente fornecidos na ração e estarem presentes nos ingredientes naturais ou sintéticos da composição da dieta. A concentração de carotenoides da dieta possui relação positiva e direta com a concentração dos mesmos nos tecidos animais associando-se, principalmente, aos lipídios pela sua composição química (Ribeiro e Seravalli, 2004).

A fonte mais importante de carotenoides na ração animal é o milho, que apresenta quantidade considerável desses elementos. Nas aves em postura esse carotenoide oriundo do milho confere a cor amarela da gema, mas não o suficiente para pigmentá-la em alaranjado ou vermelho intenso (Oliveira et al., 2007), o que normalmente ocorre quando as aves ingerem outras fontes de carotenoides na natureza

provenientes de legumes, frutas, bem como gramíneas e leguminosas, ou pela adição de carotenoides na dieta.

Os carotenoides oriundos da dieta materna aumentam a capacidade antioxidante da gema do ovo e estará disponível para o embrião em desenvolvimento (Surai, 2016) (Karadas et al., 2005b) e a progênie. Portanto, o aumento na disponibilidade de carotenoides durante a postura tem demonstrado proporcionar maior sobrevivência, condição e capacidade de desempenho da progênie (Helfenstein et al., 2008; Marri e Richner, 2014). A presença de carotenoides nos ovos tem sido associada com a maior viabilidade e eclodibilidade do ovo (Karadas et al., 2005a) e maior pigmentação da gema (Karadas et al., 2006).

1.4 Xantofilas e o extrato de *Tagetes erecta*

Na avicultura, as xantofilas sintéticas são utilizadas como suplementos alimentares para obter uma coloração ótima da pele e, especialmente, da gema do ovo. A intensidade e a cor da gema podem ser controladas pelo tipo e concentração de xantofilas da dieta. As xantofilas pertencem a uma subclasse de carotenoides e contêm pelo menos um átomo de oxigênio em sua estrutura molecular (Kurz et al., 2008). Atualmente, há crescente interesse no uso de xantofilas na alimentação animal, sendo que na alimentação humana apresenta benefícios comprovados para a saúde dos olhos bem como efeitos antioxidantes e anticâncer.

As xantofilas estão presentes em diversas plantas, dentre elas a flor de Marigold ou comumente chamada de cravo-de-defunto (*Tagetes erecta*) que possui altas concentrações deste pigmento. A *Tagetes erecta* é uma planta originária do México que apresenta flores de coloração amarela e laranja amarronzada e que, atualmente, é a única flor cultivada comercialmente como fonte de luteína (Nachtigall, 2007). Suas flores são a mais rica fonte de xantofilas, variando de 0,6 a 2,5% na matéria seca, e 88-92% das xantofilas totais são compostas por luteína e zeaxantina (Quackenbush e Miller, 1972; Rivas, 1991).

Os principais pigmentos presentes no extrato de Marigold (*Tagetes erecta* L.) são os flavonóides e os carotenoides. O extrato de Marigold contém aproximadamente 27% de carotenoides (Vasudevan et al., 1997; Wang et al., 2006), dos quais:

- 0,4% de β-caroteno,
- 1,5% de éster de criptoxantina e

- 86,1% de éster de xantofila.

O extrato de *Tagetes* contém 12 g/kg de xantofilas, dos quais 80 a 90% são de luteína, um carotenoide amarelo (Galobart et al., 2004) que representa aproximadamente metade do total de xantofilas na gema do ovo (Steinberg et al., 2000).

Os carotenoides oriundos do extrato de *Tagetes* têm sido usados na alimentação das aves de postura com o objetivo de aumentar a cor amarela de gemas de ovo (Vasudevan et al., 1997; Wang et al., 2006). A função pigmentante se dá pela deposição de carotenoides do grupo xantofila. Estes grupos estão disponíveis em alimentos como o milho e o pimentão vermelho. Há ainda carotenoides sintéticos como a cantaxantina 10% (pigmento vermelho) e a etil éster beta apo-8-caroteno (pigmento amarelo) (Garcia et al., 2002).

1.5 Metabolismo dos carotenoides

Como os carotenoides são substâncias lipossolúveis, o processo de absorção e mecanismos de transporte celular são similares ao dos lipídeos. Após a ingestão de alimentos, os carotenoides são liberados da matriz por enzimas digestivas, incluindo a lipase, e ainda emulsionados por sais biliares e fosfolipídios, também envolvidos na emulsificação de triglicerídeos da dieta e outros nutrientes lipossolúveis, como as vitaminas. Assim, os carotenoides atravessam a membrana celular via lipoproteínas e se acumulam em células de diversos tecidos. Nas poedeiras em particular são transferidos em grande quantidade para o conteúdo da gema do ovo (Faehnrich et al., 2016). Desta forma, à medida que o teor de carotenoide na ração aumenta, a sua concentração na gema do ovo aumenta na mesma proporção.

Uma vez no estômago, os carotenoides são liberados da matriz e se dissolvem na emulsão do conteúdo gástrico, passando ao duodeno para serem absorvidos na mucosa intestinal (Figura 2).

Apesar das lacunas na nossa compreensão da absorção de carotenoides e xantofilas em espécies de aves, parece provável que a maioria dos carotenoides sejam absorvidos no jejuno.

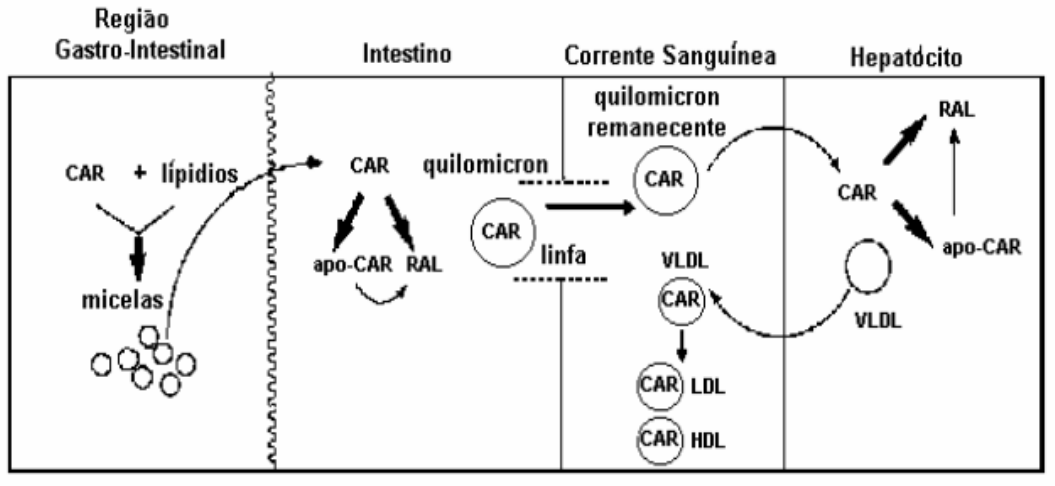

Figure 2 - Absorção, metabolismo, e transporte de carotenoides: apo-CAR, apo-carotenoide; RAL, retinal; VLDL, lipoproteína de muita baixa densidade; LDL, lipoproteína de baixa densidade; HDL, lipoproteína de alta densidade.
Adaptado de (Parker, 1996).

A solubilização de materiais insolúveis em água, incluindo carotenoides, é um passo crítico em sua digestão e absorção. Devido ao tamanho, muito pequeno das micelas mistas, elas são dispersas no ambiente aquoso do lúmen intestinal e podem se difundir para a camada glicoproteica que circunda as microvilosidades ou a borda em escova das células da mucosa, e entram em contato com as membranas celulares (Tee, 1992). Os carotenoides são transferidos das gotículas lipídicas para micelas mistas produzidas pela ação de sais biliares, fosfolipídios biliares, lipídios dietéticos e seus produtos de hidrólise (Yonekura e Nagao, 2007).

Após a solubilização em micelas mistas, os carotenoides são absorvidos pelas células intestinais e empacotados em quilomícrons. Desta forma, precisam ser incorporadas às micelas e, em seguida, os carotenoides são absorvidos por difusão passiva (Reboul et al., 2005). Entretanto, existem estudos sobre o envolvimento de um processo ativo na absorção de carotenoides, através da proteína transportadora "Scavenger Receptor class B type I" (SR-B1) que é encontrada no intestino delgado, bem como no fígado, glândulas suprarenais, ovários, placenta, rins, próstata e cérebro (Lobo et al., 2010).

As lipoproteínas do plasma servem como sistema de transporte não só para lipídeos, mas também para os carotenoides. O termo lipoproteína se refere ao conjunto de partículas esféricas complexas formadas a partir de lipídios e proteínas. Essas partículas circulam na corrente sanguínea, transportando lipídios endógenos e exógenos

no plasma. Em aves, os portomicrons - lipoproteínas produzidas pelos enterócitos - servem para transportar os lipídios do trato gastrintestinal para o fígado através da circulação portal. Lipoproteína de Muita Baixa Densidade (VLDL), Lipoproteína de Baixa Densidade (LDL) e Lipoproteína de Alta Densidade (HDL) são as principais classes de lipoproteínas do plasma das aves.

A regulação da absorção intestinal dos carotenoides parece ser mediada por um mecanismo de feedback negativo, envolvendo o fator de transcrição "intestine-specific homeobox" (ISX) (Lobo et al., 2010).

Uma vez no fígado, os carotenoides podem ser convertidos a retinoides e outros metabólitos, exercendo funções biológicas no próprio fígado ou então incorporados em lipoproteínas, e os remanescentes transportados aos tecidos extra-hepáticos. Eles são empacotados juntamente com os triglicerídeos em VLDL e transportados para o sangue, e do sangue para o tecido adiposo, uma vez que os triglicerídeos são removidos e a gordura armazenada em gotículas de gordura dentro do adipócito. A extração dos lipídeos das VLDL é acompanhada de perda de parte das apolipoproteinas, convertendo gradativamente as VLDL em LDL que transporta o colesterol para os tecidos extra-hepáticos ou de volta ao fígado como HDL. Os fatores específicos que regulam a absorção dos carotenoides pelos tecidos, sua reciclagem de volta para o fígado e excreção, não são ainda compreendidos (Canene-Adams e Erdman, 2009).

Acredita-se que a matriz alimentar seja importante para a absorção de carotenoides, pois a quantidade e o tipo de alimento determinam a secreção biliar, enquanto os sais biliares e a gordura determinam a formação de micelas (Van Vliet, 1996). O pH no lúmen intestinal também pode afetar a absorção através de um efeito sobre as cargas superficiais tanto das partículas micelares quanto da membrana celular luminal, com menor resistência à diluição em pH mais baixo (Hollander, 1981).

1.6 Uso de carotenoides em dietas de aves

Karadas et al. (2006) avaliaram os efeitos do concentrado de alfafa, tomate em pó e extrato de marigold como aditivo alimentar para codornas e verificaram que a mistura aumentou a pigmentação da gema e os níveis de carotenoides nos ovos. As concentrações de luteína, zeaxantina, licopeno e β-caroteno foram aumentados nos ovos de codornas alimentadas com dietas suplementadas com carotenoides naturais em comparação ao grupo controle.

Ainda de acordo com Karadas et al. (2006), parece provável que a luteína (xantofila) seja transferida da ração para a gema de ovo com mais eficiência, porém essa eficiência depende do tipo de carotenoide e do conteúdo da dieta. Comparando dietas contendo diferentes fontes de carotenoides, a dieta com baixo teor de carotenoides, que a luteína compreendeu 78,6% dos carotenoides totais, sua eficiência de transferência para a gema foi calculada em 38,9%. Neste estudo, a luteína foi preferencialmente e mais eficientemente depositada na gema do ovo do que qualquer outro carotenoide.

Englmaierová et al. (2013) comparando os efeitos de dietas em galinhas Isa Brown suplementadas com os carotenoides sintéticos Carophyll Red® e Carophyll Yellow® a 20 e 15 mg/kg, respectivamente, luteína a 250 mg/kg e algas *Chlorella* a 12,5 g/kg, observaram que a suplementação de ração com luteína e *Chlorella* aumentou significativamente ($P < 0,001$) a concentração de luteína (de 12,8 a 133,9 e 49,0 mg/kg de matéria seca) e zeaxantina (de 9,2 a 123,9 e 40,1 mg/kg de matéria seca) nas gemas e todos os carotenoides. A luteína e a zeaxantina representaram mais de 90% do total de carotenoides na gema, aumentando o escore de pigmentação.

A intensidade e cor (amarelo-vermelho) das gemas podem ser controlados pela concentração e tipos de carotenoides dietéticos. A deposição de carotenoides em gemas depende da sua polaridade, que é menor em carotenos não polares do que em xantofilas, que contém pelo menos um átomo de oxigênio (Englmaierová et al., 2013).

Surai et al. (2001), compararam ovos, embriões e pintos produzidos por matrizes alimentadas com dieta à base de milho, rica em carotenoides, especialmente luteína e zeaxantina (11,8 mg de carotenoides/kg) e à base de trigo (5,6 mg de carotenoides/kg). Os ovos provenientes de galinhas alimentadas com dieta à base de milho apresentaram maiores ($P \leq 0,01$) concentrações de beta + gama-tocoferol, carotenoides totais, luteína e zeaxantina, assim como os tecidos dos pintos nascidos destes ovos. Os autores concluíram que a dieta da matriz tem importante função na formação do sistema antioxidante durante o desenvolvimento embrionário e que a dieta à base de milho aumenta o potencial antioxidante da gema do ovo e dos tecidos embrionários, quando comparada à dieta a base de trigo.

1.7 Formação do ovo, deposição de carotenoides e processos de oxidação em aves

A formação de ovos é um processo complexo, envolvendo o aumento substancial no metabolismo de lipídios e proteínas no fígado e outros órgãos. A cor da gema é completamente determinada pelos tipos e quantidades de carotenoides no alimento e pela capacidade da ave em absorver e assimilar esses pigmentos.

O sistema reprodutivo da galinha é um sistema muito complexo que pode produzir um ovo em 24 a 25 horas. Um ovo é composto pela gema (30 - 33%), albumina (60%) e casca (9 - 12%). A ovulação geralmente ocorre a cerca de 30 minutos após a postura do ovo (Lovell et al., 2003).

Sabe-se que a ave reprodutora nasce com um ovário e este pode conter milhares de minúsculos óvulos ou futuras gemas. Quando a franga atinge a maturidade sexual, os óvulos começam a se desenvolver, um de cada vez. O ovário contém óvulos em vários estágios de desenvolvimento, desde óvulos brancos muito pequenos até óvulos amarelos quase maduros, prontos para serem ovulados. Cada gema é incluída em seu próprio saco chamado folículo. A gema é mantida intacta pela membrana vitelina circundante.

Os ovos são constituídos por aproximadamente 11% de lipídeos, localizados principalmente na gema, que representa 33 a 35% do peso do ovo. A gema é rica em lipídios em geral, vitaminas lipossolúveis como as vitaminas E e A e por vários carotenoides (Griffin et al., 1984). Os lipídeos da gema desempenham papel importante no desenvolvimento do embrião, sendo utilizados como fonte de energia, ácidos graxos e vitaminas lipossolúveis. A gema é composta de 30 a 34% de gorduras, contendo colesterol (5% do total gorduroso), triglicerídeos (66%), fosfolipídios (28%) e ácidos graxos livres (1%), sendo que na porção lipídica, as maiores concentrações são de ácidos graxos insaturados (Sarcinelli, 2007).

As proteínas e lipídios da gema são sintetizados no fígado mediante a ação do estrogênio, e essas proteínas são levadas para dentro do oócito por um receptor de lipoproteína específico (Stifani et al., 1990). As lipoproteínas de densidade muito baixa (VLDL) são o principal sistema de entrega de carotenoides no oócito em desenvolvimento, processo induzido por estrógenos (Walzem et al., 1999). Sendo assim, a gema do ovo é uma fonte rica de carotenoides, porém sua coloração depende da quantidade de xantofilas, absorvidas pela ave que se alimenta de milho ou ração (Garcia et al., 2002).

Os carotenoides responsáveis pela cor do grão de milho são também os pigmentos responsáveis pela coloração da gema do ovo. Os grãos de milho, especialmente amarelo-alaranjados, destacam-se como fonte de carotenoides, estando presentes nesses grãos as xantofilas (luteína, zeaxantina e β-criptoxantina), que perfazem a cerca de 90% do total dos carotenoides, além dos carotenos (β-caroteno, α-caroteno e β-zeacaroteno) que somam os 10% restantes (Kurilich e Juvik, 1999; Egesel et al., 2003). Nos milhos normais, a concentração total de carotenoides varia de 0,15 a 33,11µg/g, distribuídos essencialmente no endosperma do grão (Kurilich e Juvik, 1999). A distribuição dos carotenoides no grão seco é 74 a 86% no endosperma vítreo, 9 a 23% no endosperma farináceo, 2 a 4% no germe e 1% no farelo; sendo o endosperma vítreo presente em maior proporção (46 a 54%) que o farináceo (28 a 36%) (Blessin et al., 1963).

O desenvolvimento embrionário das aves está baseado nos nutrientes disponíveis no ovo e na interação do ovo e do embrião com o ambiente em que este se desenvolve. Embora o estado nutricional de um animal seja baseado principalmente em sua exposição a nutrientes dietéticos, o papel da nutrição materna também é relevante. O embrião em desenvolvimento depende de nutrientes maternos como substratos para proliferação e diferenciação celular. Além disso, a contribuição dos nutrientes maternos pode afetar a fisiologia do animal mais tarde na vida, ou pode afetar tanto a embriogênese quanto à função futura.

O conteúdo e o perfil de carotenoides na gema são determinados, em parte, pela ingestão dietética, mas também são influenciados por fatores fisiológicos que podem apresentar variação filogenética (Schaeffer et al., 1988; Surai e Speake, 1998).

Em ovos férteis, a oxidação dos lipídios da gema não é citada como causa dos efeitos negativos do armazenamento sobre o desenvolvimento embrionário e o rendimento de incubação. No entanto, para a qualidade de ovos de consumo, as explicações se baseiam nas mudanças físicas que ocorrem no ovo, especialmente no albúmen, tais como aumento do pH tornando mais liquefeito (Lapão et al., 1999; Xavier et al., 2008) e redução do índice de gema em razão do enfraquecimento da membrana perivitelina (Kirunda e McKee, 2000).

O perfil de carotenoides presente na gema do ovo é altamente dependente do tipo de carotenoides presentes na dieta que foi consumida pela ave (Surai et al., 1998). Outros fatores também são importantes determinantes do perfil nutricional da gema, tais como, a eficiência de absorção dos diferentes carotenoides no intestino e na medida que

os carotenoides são convertidos em vitamina A na mucosa intestinal ou no fígado (Hamilton, 1992). É conhecido que o β-caroteno não é acumulado nos tecidos de galinhas ou de seus ovos (Brush, 1990; Hudon, 1994).

A ingestão de carotenoides pelas aves pode aumentar a capacidade antioxidante total no organismo e sua transferência para o conteúdo da gema leva a proteção do conteúdo lipídico da mesma, protegendo o ovo dos efeitos prejudiciais das espécies reativas de oxigênio e dos RL (Stahl e Sies, 2003). Assim, os carotenoides podem ajudar a proteger a ave em desenvolvimento contra o dano oxidativo, particularmente durante os períodos sensíveis de eclosão e a vida pós-eclosão inicial (Surai et al., 1996). Carotenoides em gema de aves são transferidos para o embrião em desenvolvimento, e parecem contribuir para a capacidade antioxidante dos tecidos, possivelmente pela sinergia com a vitamina E (Surai e Speake, 1998).

Nos processos de oxidação que ocorrem nos ovos, os lipídios presentes na gema são oxidados em RL (Ren et al., 2013). A reação de RL com ácidos graxos poli-insaturados inicia um processo em cadeia conhecido como peroxidação lipídica. Os ácidos graxos poli-insaturados presentes na gema dos ovos com cadeias longas são particularmente suscetíveis à peroxidação (Pappas et al., 2005; Mohiti-Asli et al., 2008), e altera a estrutura das membranas celulares, provocando modificações na estrutura dos aminoácidos, mudanças nas atividades enzimáticas e atingindo o DNA (Lima e Abdalla, 2001).

Quando ocorre oxidação na gema do ovo, os lipídios são oxidados em RL, como radicais de oxigênio e radicais de peróxido que atacam o material peroxidável disponível produzindo hidroperóxidos (Ren et al., 2013). A reação de RL com ácidos graxos inicia um processo em cadeia conhecido como peroxidação em sistemas vivos e rancidez oxidativa em alimentos (Rocha et al., 2013).

1.8 Considerações finais

Dietas enriquecidas com substâncias antioxidantes como os carotenoides, possuem o potencial de melhorar o status oxidativo do metabolismo geral das aves e dos ovos. Esses efeitos se refletem na condição de saúde e bem-estar das matrizes e da progênie, contribuindo para a produção de ovos férteis de qualidade e influenciando o desenvolvimento embrionário e o desempenho do pintinho pós-eclosão com melhor qualidade.

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II. OBJETIVOS GERAIS

Avaliar a inclusão de níveis de extrato de *Tagetes erecta* na dieta de matrizes de codornas *Coturnix coturnix japonica*, sobre o desempenho produtivo, qualidade de ovos, desempenho de incubação e progênie, variáveis bioquímicas e antioxidantes.

OBJETIVOS ESPECÍFICOS

Artigo I:

Identificar os possíveis efeitos da suplementação do extrato de *Tagetes erecta* na dieta de reprodutores de codornas japonesas sobre a produção, a qualidade de ovos e as variáveis bioquímicas séricas e oxidativas.

Artigo II:

Identificar os possíveis efeitos da suplementação do extrato de *Tagetes erecta* na dieta de reprodutores de codornas japonesas sobre o processo de incubação, a qualidade, desempenho produtivo, capacidade antioxidante e peroxidação lipídica de pintinhos.

III. PERFORMANCE, EGG QUALITY, AND SERUM AND OXIDATIVE PROFILES OF JAPANESE QUAIL (*COTURNIX COTURNIX JAPONICA*) BREEDERS SUPPLEMENTED WITH *TAGETES ERECTA* EXTRACT

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Title of the manuscript: III. Performance, egg quality, and serum and oxidative profiles of Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with *Tagetes erecta* extract

Abstract

Objective: This study aimed to assess the effects of supplementation with *Tagetes erecta* extract on the performance, egg quality, and serum and oxidative profiles of Japanese quail (*Coturnix coturnix japonica*) breeders.

Methods: A total of 480 quail breeders were randomly distributed into five treatments (diets), with 12 replications. Each experimental unit consisted of six females and two males. Birds were fed a basal diet supplemented with 0, 1, 2, 3, or 4 g/kg *T. erecta* extract.

Results: Productive performance and egg quality were not influenced ($P > 0.05$) by *T. erecta* extract supplementation. Yolk color was affected by supplementation: L* decreased and a* increased linearly with increasing extract concentrations. β-carotene and xanthophyll contents in diets and egg yolk also increased with *T. erecta* extract concentration. Serum analysis showed that supplementation exerted a quadratic effect on albumin levels in males, with the highest concentration (2.39 g/dL) estimated to be achieved by supplementation with 1.87 g/kg *T. erecta* extract. In females, serum triglycerides decreased linearly with increasing extract concentrations; supplementation with 1 g/kg extract reduced serum triglycerides by 60.92 mg/dL. Serum DPPH antioxidant capacity increased linearly ($P < 0.05$) in females, but no effects ($P > 0.05$) were observed on conjugated diene formation or ABTS scavenging activity. Liver superoxide dismutase activity had a quadratic relationship with extract concentration in females; the highest activity (0.0471 U/mg) was estimated to be achieved

with 2.61 g/kg extract. Expression of *SOD1* and *GPX7* genes in the vaginal mucosa was not influenced ($P > 0.05$) by supplementation.

Conclusion: Dietary concentrations of *T. erecta* extract generally used to alter yolk color (<4 g/kg diet) are not sufficient to enhance the antioxidant capacity of the serum, liver, or vaginal mucosa of Japanese quail breeders raised under optimal conditions for egg production.

Keywords: Antioxidant; Productive indices; Egg quality; Lipid oxidation.

Introduction

The nutrition status of poultry breeders directly influences egg composition, embryonic development, and chick post-hatch performance. Quail breeders have a lower production cost and faster development than broiler breeders and are, therefore, more indicated for investigation of the effects of antioxidant supplementation on physiological parameters and embryonic development.

Supplementation of poultry diets with antioxidant compounds seems to be an efficient strategy to improve the oxidative stability of poultry products such as eggs and meat (Yesilbag et al., 2013). Plant extracts, essential oils, and phytogenic additives are commonly added to breeder and chick diets because of their high content of biologically active compounds, including polyphenols, carotenoids, and vitamins. Carotenoids are a class of natural, fat-soluble, polyunsaturated pigments found mainly in plants, algae, and photosynthetic bacteria (Raaman, 2006). The antioxidant properties of carotenoids and the capacity to interact with biological membranes derive from their chemical structure (Gammone et al., 2015). Carotenoids play a role in the regulation of cell growth, gene expression, and immune response (Rao and Rao, 2007); (Bertram, 2009).

Carotenoids containing oxygenated functional groups are denominated xanthophylls. Examples include lutein and zeaxanthin (Zaripheh and Erdman, 2002); Almeida-Muradian and Penteado, 2003). Such compounds are highly efficient in scavenging and stabilizing free radicals. Extracts obtained from *Tagetes erecta* petals are rich in lutein (Rodriguez and Mabry, 1985), the major carotenoid in *T. erecta*-based products (McGEACHIN and BAILEY, 1995), and lutein esters, which account for >90% of the flower's pigments (Quackenbush and Miller, 1972)(Barzana et al., 2002). Analysis of saponified extracts of *T. erecta* flowers revealed the presence of α-carotene, β-carotene, and phytofluene (Hadden et al., 1999).

Antioxidants can mitigate the negative effects of oxidative stress, which impairs reproduction, normal growth, and immune responsiveness, by balancing the levels of pro- and antioxidants (Costantini and Møller, 2009)(Surai, 2007). During laying, quail are more prone to metabolic disorders and free radical damage because of the intense metabolic activity stimulated by continuous egg production and environmental stress (Zou et al., 2007). Although various studies have been carried out on the effects of antioxidants on breeder, layer, and meat poultry, there are still many gaps in our knowledge of physiological responses to different antioxidant substances. This study aimed to investigate the effects of supplementation of Japanese quail (*Coturnix coturnix japonica*) breeders with *T. erecta* extract on productive performance, egg quality, and serum and oxidative profiles.

Material and methods

All experimental procedures were approved by the Animal Ethics Committee of the State University of Maringá, Paraná, Brazil (protocol no. 3486021017).

Animals, housing conditions, and handling

One-day-old Japanese quail chicks of both sexes were acquired from a commercial breeding facility (Vicami®, Assis, São Paulo, Brazil) and reared until 45 days of age in 2.5 m^2 boxes in a conventional pen. After this period, the birds were moved to galvanized wire cages (25 × 39 cm) equipped with a nipple drinker at the top and a trough feeder at the front and maintained in a laying house.

Each cage contained six females and two males, randomly allocated by body weight (mean of 146.33 g for females and 121.00 g for males) and egg production (91.16%). Food and water were provided *ad libitum*, and the birds were kept under a 17 h light (natural + artificial)/7 h dark photoperiod. Males and females sharing a cage received the same diet. The

temperature and relative humidity within the laying house were measured daily using a portable hygrometer. The daily maximum and minimum temperatures were 29.72 and 16.57°C, respectively, and the maximum and minimum relative humidity was 60.76 and 40.46%, respectively.

Experimental design and diets

A total of 480 Japanese quail breeders aged 12 weeks were distributed in a completely randomized design with five treatments (diets) and 12 replications. Each cage containing six females and two males was treated as an experimental unit. The birds were fed a basal diet (T_0) or a basal diet supplemented with 1 (T_1), 2 (T_2), 3 (T_3), or 4 (T_4) grams of *T. erecta* extract per kilogram of diet (Table 1). The experimental period was 12 weeks, comprising 4 production cycles of 28 days.

For adaptation, birds were placed on experimental diets 14 days before initiation of the experiment. The diets were based on corn and soybean meal and met the nutritional requirements of quail layers, according to Rostagno et al. (2017). To obtain five isoenergetic and isonitrogenous diets, we replaced the necessary amount of inert (kaolin) by a commercial product based on *T. erecta* extract (0, 1, 2, 3 and 4 g/kg for diets T_0, T_1, T_2, T_3, and T_4, respectively). The commercial extract, produced by solvent extraction of *T. erecta* petals, contains a guaranteed level of 20 mg xanthophyll/kg. Fresh feed was prepared every 15 days and stored in dark bags in a dry environment protected from light.

Productive performance, egg quality, and color parameters

Productive performance was evaluated in terms of feed intake (g/bird/day), feed conversion (kg feed/kg dozen eggs, g feed/g egg, and g feed/egg mass), egg production, dozen egg

production, and egg mass production. Egg quality was assessed by egg weight, specific gravity, Haugh unit, yolk index, shell thickness, and eggshell, albumen, and yolk percentages.

Eggs from each replication (cage) were collected in the last three days of each production cycle. Intact eggs were weighed to the nearest 0.01 g and then analyzed for specific gravity in saline solutions with densities varying from 1.060 to 1.090 g/cm^3 at 0.005 g/cm^3 intervals. Three eggs per experimental unit were used for analysis of eggshell, albumen, and yolk percentages.

Internal egg quality was assessed by analyzing albumen and yolk heights and eggshell thickness. Three eggs from each experimental unit were weighed separately on a semi-analytical scale (0.001 g resolution) and then broken on a flat, smooth glass surface. Albumen and yolk heights were measured to the nearest millimeter using a digital caliper. Albumen height (h, expressed in millimeters) and egg weight (w, expressed in grams) were used to calculate the Haugh unit (HU) (Haugh, 1937) by the equation $HU = 100 \log (h + 7.57 - 1.7w^{0.37})$. The yolk diameter was determined by using a manual caliper (0.05 mm resolution), and mean values were used for calculation of the yolk index (yolk height divided by yolk diameter).

Eggshell percentage was determined as the ratio of eggshell weight to egg weight. For this, eggshells were collected, washed with water, dried at room temperature for 72 h, and weighed using a semi-analytical scale (0.001 g resolution). Subsequently, shell thickness was determined at three points in the central portion of each eggshell using a digital micrometer (Mitutoyo®, Japan).

Yolk color was determined using a Minolta CR-400 colorimeter calibrated against a white surface according to Bible and Singha (1993) and Mutschler et al. (1992). Results are expressed in CIELab values: a* (redness/greenness, ranging from +a* to −a*), b*

(yellowness/blueness, ranging from +b* to −b*), and L* (lightness, ranging from 100 to 0, which represent white and black, respectively.

Carotenoid content in egg yolk and diets

Egg yolks from 29-week-old breeders and experimental diets were evaluated for β-carotene and xanthophyll contents by a specialized company. Samples were subjected to extraction with organic solvents, saponification, carotenoid separation by column chromatography, and absorbance measurement using a UV/Vis spectrophotometer, according to method no. 9 of Sindirações (2009).

Serum and tissue sampling

At the end of the experimental period, 10 females and 10 males per treatment were selected on the basis of body weight (±5% of the mean body weight of replicates) and fasted for 6 h. Blood samples were collected and centrifuged, and the serum was stored at −20°C until analysis. Of the 10 females selected per treatment, 5 were euthanized by intra-abdominal injection of barbituric (25 mg/kg body weight sodium thiopental–lidocaine). Anesthesia was determined by immobility and absence of palpebral and corneal reflex, and then birds were killed by cervical dislocation. Liver and vaginal mucosa specimens were collected and stored in liquid nitrogen until analysis.

Serum analyses

Serum albumin, total protein, cholesterol, and triglycerides were determined (n = 10) by a colorimetric method using commercial kits (Gold Analisa Diagnóstica Ltd., Minas Gerais, Brazil) as per the manufacturer's instructions.

Serum antioxidant activity

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was carried out using female serum samples (n = 5) according to the method of (Re et al., 1999), with modifications. ABTS cation radical was formed by incubating 7 mM ABTS with 140 mM potassium persulfate in the dark for 16 h at room temperature. ABTS cation radical solution was then diluted with ethanol to an absorbance of 0.70 ± 0.02, and 1960 µL of this solution was mixed with 40 µL of serum. Absorbance was read at 734 nm after 6 min, and the antioxidant activity (%) was calculated using Eq. (1):

$$ABTS = (1 - Abs/Abs_0) \times 100 \quad (1)$$

where Abs is the sample absorbance at 6 min and Abs_0 is the sample absorbance at time 0.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was also used to determine the serum antioxidant capacity in females (n = 5), following the method described by (Li et al., 2009), with modifications. Serum aliquots (150 µL) were mixed with 2.85 mL of 60 µM DPPH solution and incubated in the dark for 30 min. Absorbance was read at 515 nm, and antioxidant activity (%) was calculated using Eq. (2):

$$DPPH = (1 - Abs/Abs_0) \times 100 \quad (2)$$

where Abs is the sample absorbance at 30 min and Abs_0 is the sample absorbance at time 0. The result was expressed as half maximal inhibitory concentration (IC_{50}, the lowest concentration required to inhibit DPPH activity by 50%).

Determination of serum lipid peroxidation

Serum levels of conjugated diene hydroperoxides were determined as a measure of lipid peroxidation using copper ion as oxidant (Gobert et al., 2009). Briefly, 20 µL of serum, 20 µL of phosphate buffered saline (PBS), and 2 mL of 25 µm copper sulfate in PBS (pH 7) were added to a cuvette. Absorbance was read on a UV-Vis spectrophotometer at 37°C and 1 min

intervals for 4 h. Results were analyzed according to (Pinchuk and Lichtenberg, 2002). The maximum oxidation rate (V_{max}), optical density at the maximum conjugated diene accumulation (OD_{max}), and time to reach V_{max} (T_{max}) were determined from the absorbance curve.

Liver superoxide dismutase (SOD) activity

Liver SOD activity (n = 5) was determined by estimating the ability of the enzyme to inhibit pyrogallol autoxidation in alkaline medium at 420 nm (MARKLUND and MARKLUND, 1974). One unit (U) of SOD activity was defined as the concentration needed to inhibit autoxidation by 50%. Results are expressed as U/mg protein.

Analysis of gene expression in vaginal mucosa

For analysis of gene expression, five samples of vaginal mucosa were collected, immediately frozen in liquid nitrogen, and stored in a freezer at −80°C until RNA extraction. Total RNA was extracted from 100 mg samples using 500 µL of Trizol (Invitrogen, USA), according to the manufacturer's instructions. Samples were homogenized by vortexing and incubated at room temperature for 5 min. Then, 100 µL of chloroform was added, and samples were manually homogenized for 15 s, incubated at room temperature for 3 min, and centrifuged at 12 000 × g and 4°C for 15 min. The aqueous phase was transferred to a new tube, and 250 µL of isopropanol was added. The mixture was left to rest for 10 min at room temperature, manually homogenized, and centrifuged at 12 000 × g and 4°C for 15 min. After the supernatant was discarded, the precipitate was washed with 500 µL of 75% ethanol. Samples were centrifuged at 7 500 × g for 5 min, the supernatant discarded, the pellet left to dry for 15 min, resuspended in RNase-free ultrapure water, and incubated at 60°C for 15 min. RNA concentration was measured at 260 and 280 nm using a NanoDrop 2000 spectrophotometer

(Thermo Fisher Scientific, USA). Total RNA samples were treated with DNase I (Invitrogen Corporation, USA) for removal of genomic DNA, as per the manufacturer's instructions.

Complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen Corporation, USA) according to the manufacturer's instructions. To a sterile, RNase-free tube were added 8 µL of total RNA, 1 µL of 50 µM oligo(dT) 20 primer, and 1 µL of 10 mM dNTP mix. The reaction was incubated at 65°C for 5 min, placed on ice for 1 min, and then mixed with a solution containing 2 µL of RT buffer (10×), 4 µL of $MgCl_2$ (25 mM), 2 µL of DTT (0.1 M), 1 µL of RNaseOUT (40 U/µL), and 1 µL of SuperScript III RT (200 U/µL). cDNA synthesis was performed by incubation at 50°C for 50 min and 85°C for 5 min. Upon completion of the reaction, the mixture was immediately placed on ice. Then, 1 µL of RNase H was added to each tube, and samples were incubated at 37°C for 20 min. cDNA samples were stored at −20°C until use.

Real-time polymerase chain reactions (PCR) were carried out in duplicate using PowerUp SYBR Green Master Mix (Applied Biosystems, USA) and a StepOnePlus Real-Time PCR system (Applied Biosystems, USA). The reaction mixture (final volume of 20 µL) was composed of PowerUp SYBR Green, ultrapure water, primers, and cDNA. The primers for superoxide dismutase (*SOD1*) and glutathione reductase (*GPX7*) genes were designed from sequences for Japanese quail deposited at www.ncbi.nlm.nih.gov (Table 2). The β-actin gene from *Gallus gallus* was used as endogenous control.

Statistical analysis

All statistical analyses were performed using SAS version 2010. Diet effects were assessed by analysis of variance and regression. The level of significance was set at $P < 0.05$.

Results

Overall, supplementation with *T. erecta* extract did not affect ($P > 0.05$) the performance or egg quality of Japanese quail breeders (Table 3). The observed productive variables were consistent with literature data for the same quail species and age.

Yolk color was significantly influenced ($P < 0.05$) by *T. erecta* extract concentration (Table 4). Luminosity decreased with increasing supplementation levels ($L^* = 43.6813 - 0.511x$), whereas redness/greenness showed the opposite behavior ($a^* = -0.6487 + 0.7922x$). Supplementation had no effect ($P > 0.05$) on blueness/yellowness.

β-Carotene and xanthophyll contents in experimental diets and egg yolk increased with increasing extract concentrations (Table 5). *T. erecta* extract supplementation had no effect ($P > 0.05$) on serum albumin, cholesterol, or ABTS scavenging activity in females or on serum cholesterol or triglycerides in males (Tables 6, 7). Serum total protein in females and males and DPPH antioxidant capacity in females increased linearly ($P < 0.05$) with supplementation. *T. erecta* extract concentration exerted quadratic effects ($P < 0.05$) on serum albumin in males; the highest albumin level (2.39 g/dL) was estimated to be reached with 1.87 g/kg extract. Serum triglycerides decreased linearly ($P < 0.05$) in females with *T. erecta* extract supplementation: 1 g/kg extract reduced serum triglyceride levels by 60.92 mg/dL.

Serum analysis of the formation of lipid peroxidation products (conjugated dienes) (Table 7) revealed that *T. erecta* extract supplementation did not affect ($P > 0.05$) OD_{max}, V_{max}, or T_{max} during copper-induced oxidation.

Figure 1 shows the liver SOD activity of breeders fed diets containing different concentrations of *T. erecta* extract. Extract concentration had a quadratic effect on SOD activity (SOD activity $= -0.00049 + 0.0365x - 0.0070x^2$). It was estimated that the highest SOD activity (0.0471 U/mg) can be obtained by supplementation with 2.61 g/kg extract.

SOD1 and *GPX7* expression levels in the vaginal mucosa of breeder quail are shown in Figure 2. Relative gene expression was not altered by supplementation ($P > 0.05$).

Discussion

Several studies on the effects of *T. erecta*-based products on broilers and breeders found that supplementation did not significantly alter or improve productive performance (Wang et al., 2017)(Karadas et al., 2016). (Alay and Karadas, 2017) supplemented quail breeder diets with synthetic and natural carotenoids (including *T. erecta* extract) and did not observe differences ($P > 0.05$) in egg production, egg weight, feed intake (g/bird/day), or feed conversion (g/g egg mass). By increasing the dietary levels of lutein of layer hens, (Leeson and Caston, 2004) observed no effects on egg production, egg weight, eggshell quality, or feed intake ($P > 0.05$).

(Santos-Bocanegra et al., 2004) used natural (*T. erectus* and *Capsicum* spp.) and synthetic (citranaxanthin, canthaxanthin, and β-apo-8-carotenoic acid ethyl ester) sources of xanthophylls to enhance the yellow color of ISA Brown chicken eggs. No differences ($P > 0.05$) were observed in egg production, feed intake, or feed conversion between treatments, but yolk color differed significantly ($P < 0.001$) depending on the pigments and concentrations used.

Although *T. erecta* extract supplementation did not significantly influence productive performance, it promoted a slight increase in egg production. *T. erecta* extract contains compounds that stimulate the secretion of pancreatic and gastric enzymes, possibly increasing nutrient digestibility, intestinal function, and, consequently, egg production (Suresh and Srinivasan, 2007).

Angeles and Scheideler (1998) compared the effects of two basal diets based on corn gluten and alfalfa meal supplemented with 45 or 60 ppm xanthophyll (from carophyll yellow and carophyll red). The authors found that yolk color, but not animal performance, was

significantly influenced after eight weeks of supplementation. (Galobart et al., 2004), Moura et al. (2011), and (Garcia et al., 2015) reported that natural pigments did not influence the quality of poultry eggs, only yolk color.

The decrease in lightness with *T. erecta* extract concentration was probably associated with the extract's high content of xanthophylls, 80–90% of which is the yellow carotenoid lutein (Moura et al., 2011). Thus, the more intense the yolk color, the lower the lightness. This relationship agrees with the results obtained by (Skřivan et al., 2016), who supplemented layer chicken diets with *T. erecta* extract (0, 150, 350, 550, 750, and 950 mg/kg) and observed a decrease in yolk lightness. Similarly, (Valentim et al., 2019) added natural and synthetic pigments to the diet of Black Avifran layer chickens and found that supplementation with 0.8% *T. erecta* extract increased the chroma value (78.84) of yolk, leading to increased yellowness.

The increase in yolk redness/greenness can be explained by the miscibility of lutein fatty acid esters with vegetable oils, generating a yellow gold to orange color (Carolina et al., 2009). (Alay and Karadas, 2017) found that *T. erecta* extract supplementation increased yolk redness, decreased lightness, and did not influence blueness/yellowness.

The yellow color of yolk stems mainly from the presence of xanthophylls (lutein and zeaxanthin) and β-carotene traces. The concentration of these compounds in yolk varies according to dietary pigment sources (Mínguez-Mosquera et al., 2002). Eggs normally contain 0.3–0.5 mg xanthophylls, more than half of which is lutein (Steinberg et al., 2000). Most compounds present in the egg are metabolized in the liver, accumulated in the blood, and then transported to the ovary, where deposition of fat-soluble compounds, lipids, phospholipids, cholesterol, and carotenoids occur, conferring a yellow-orange color to yolk (Lopes et al., 2011).

Carotenoid and xanthophyll concentrations in egg yolk were correlated with yolk color. The higher the concentration of *T. erecta* extract in the diet, the higher the deposition of carotenoids and xanthophylls in egg yolk. In birds, dietary pigments are absorbed from the intestinal lumen, and carotenoids are transported into cells together with lipids via membrane lipoproteins. These pigments can accumulate in the cells of lipid-rich tissues, such as egg yolk (Faehnrich et al., 2016).

The serum composition of birds differs according to sex (Rezende, 2017), which explains the differences observed in the present study between female and male quail breeders. *T. erecta* extract supplementation exerted significant effects on serum albumin in males only. Total serum protein levels (2.63 to 4.55 g/dL) were similar to those reported by Kaneko et al. (2008) for healthy birds (2.5 to 5.6 g/dL). The reduction in serum triglyceride in females can be explained by the presence of the flavonoid quercetagetin in *T. erecta* extract. One of the bioactive properties of this compound is the ability to reduce serum triglyceride levels (Hammoda, 2004; (Wang et al., 2016).

Antioxidant assays (ABTS and DPPH) demonstrated the antioxidant effects of *T. erecta* extract supplementation in female quail breeders. The lower the concentration of an antioxidant compound needed to reduce the initial DPPH level by 50%, the greater its antioxidant capacity (Fernandes, 2017). Extract concentrations of 1 to 2 g/kg diet were the most efficient in increasing serum antioxidant capacity, as lower concentrations were needed to reduce DPPH activity by 50%.

Estrogens contribute to the high levels of serum triglycerides in layer birds during egg formation (Sturkie, 1976). Polyunsaturated fatty acids are oxidized via hydroperoxide formation and reaction with double bonds, leading to the generation of conjugated dienes (Jadhav et al., 1996). We determined the concentration of conjugated dienes as a measure of

serum lipid peroxidation. However, supplementation with *T. erecta* extract was not sufficient to reduce or prevent lipid peroxidation in females.

Liver SOD activity had a quadratic relationship with *T. erecta* extract concentration, demonstrating that lutein probably prevented lipid peroxidation. In general, the results suggest that *T. erecta* extract supplementation stimulated antioxidant responses in the liver to counteract oxidative-induced damage. Thus, dietary supplementation with *T. erecta* extract at all concentrations tested contributed to increasing liver SOD activity.

Gong et al. (2012) found a defatted alcoholic extract of *T. erecta* residue to contain the following antioxidant compounds: gallic acid, gallicin, quercetagetin, 6-hydroxykaempferol-*O*-hexoside, patuletin-*O*-hexoxide, and quercetin. Quercetagetin was considered to have the highest antioxidant capacity. The rich antioxidant composition of *T. erecta* extracts partially explains the results obtained in the present study for serum antioxidant and liver SOD activities.

The vaginal mucosa of quail breeders is also susceptible to oxidative stress. Tubular glands are responsible for storing sperm after copulation. The high levels of polyunsaturated fatty acids in tubular glands predispose vaginal tissues to the action of free radicals. Breque et al. (2003) stated that the uterovaginal region has a complex defense mechanism by which antioxidant enzymes such as GPx and SOD are activated; their activity is higher than that of liver antioxidants. PCR analysis of vaginal mucosa samples showed that *T. erecta* extract supplementation had no effect on *GPX7* or *SOD1* gene expression. However, we believe that higher concentrations of *T. erecta* extract might produce antioxidant-stimulating effects, contributing to the maintenance of sperm survival in tubular glands and, consequently, quail fertility.

Conclusion

Supplementation with *T. erecta* extract increased xanthophyll levels in diets and egg yolk. Extract concentrations of up to 4 g/kg diet, generally used to alter yolk color, were not sufficient to enhance the antioxidant capacity of the serum, liver, or vaginal mucosa of Japanese quail breeders raised under optimal conditions for egg production.

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526

Table 1 - Ingredient composition, calculated nutrient content, and energy value of the basal diet fed to Japanese quail (*Coturnix coturnix japonica*) breeders

Ingredient	**%**
Maize	61.30
Soybean meal	28.88
Limestone	6.78
Soybean oil	0.21
Dicalcium phosphate	1.11
Vitamin–mineral premix[1)]	0.40
Sodium chloride	0.27
DL-Methionine (98.5 %)	0.25
L-Lysine (76.5%)	0.38
Inert[2)]	0.40
Calculated composition	
Crude protein (%)	18.92
Metabolizable energy (kcal/kg)	2800
Calcium (%)	2.99
Available phosphorus (%)	0.309
Sodium (%)	0.147
Digestible lysine (%)	1.149
Digestible methionine (%)	0.517
Digestible methionine + cysteine (%)	0.942

527 [1)] Provided per kilogram of product: 2 500 000 IU vitamin A, 500 000 IU vitamin D3, 5 000 IU vitamin E, 625
528 mg vitamin B1, 1 500 mg vitamin B2, 1 250 mg vitamin B6, 5 000 IU vitamin B12, 750 mg vitamin K3, 3 000
529 mg calcium pantothenate, 6 000 mg niacin, 250 mg folic acid, 50.0 mg biotin, 75 g choline, 1 000 mg antioxidant
530 (butylated hydroxytoluene), 13 g zinc, 13 g iron, 15 g manganese, 3 000 mg copper, 50 mg cobalt, 250 mg
531 iodine, and 63 mg selenium.

532 [2)] Kaolin.

533

Table 2 - Primers used for polymerase chain reaction amplification

Gene	Annealing temperature	Primer sequence
SOD1	60°C	F: GGAGTGGCAGAGGTAGAAATAG R: AGGTCCAGCATTTCCAGTTAG
GPX7	60°C	F: TGGTGCCTCCTTTCCTATGT R: GTTCCAGGTTGGTTCTTCTCCT
β-Actin	60°C	F: GCCAACAGAGAGAAGATGAC R: CACCAGAGTCCATCACAATAC

534 F, forward primer; R, reverse primer.

535

Table 3 - Productive performance and egg quality of Japanese quail (*Coturnix coturnix japonica*) breeders fed diets supplemented with different concentrations of *Tagetes erecta* extract

Parameter	**Extract concentration (g/kg diet)**					**SEM**	***P*-value**	
	0	**1**	**2**	**3**	**4**		**Linear**	**Quadratic**
Feed intake (g/bird/day)	27.10	26.58	26.64	26.41	26.28	0.110	0.232	0.586
Feed conversion (kg/dozen eggs)	0.37	0.35	0.35	0.35	0.34	0.004	0.129	0.363
Feed conversion (g/g egg)	2.28	2.24	2.26	2.25	2.26	0.014	0.516	0.586
Feed conversion (g/g egg mass)	2.50	2.37	2.38	2.41	2.40	0.067	0.095	0.159
Egg production (%)	91.43	94.20	95.00	93.56	95.02	0.429	0.047	0.158
Egg weight (g)	11.88	11.89	11.78	11.73	11.69	0.046	0.657	0.997
Daily egg mass production (g/bird/day)	10.86	11.19	11.20	10.98	11.04	0.022	0.159	0.174
Albumen (%)	61.65	61.63	61.52	61.67	61.51	0.083	0.948	0.961
Yolk (%)	30.88	30.90	30.85	30.83	30.87	0.082	0.868	0.897
Eggshell (%)	7.48	7.60	7.68	7.63	7.69	0.032	0.140	0.347
Specific gravity	1.070	1.072	1.072	1.071	1.072	0.000	0.144	0.257
Haugh unit	87.69	88.41	87.66	88.22	88.12	0.237	0.781	0.864
Yolk index	0.47	0.46	0.47	0.47	0.47	0.001	0.709	0.519
Shell thickness (mm)	0.20	0.21	0.21	0.21	0.21	0.001	0.114	0.237

536 SEM, standard error of mean.

537

538

Table 4 - Color parameters of eggs from Japanese quail (*Coturnix coturnix japonica*) breeders fed diets supplemented with different concentrations of *Tagetes erecta* extract

Parameter	**Extract concentration (g/kg diet)**					**SEM**	***P*-value**	
	0	**1**	**2**	**3**	**4**		**Linear**	**Quadratic**
L*	43.72	43.19	42.57	42.10	41.71	0.138	0.016	0.611
a*	−0.87	0.34	1.07	1.77	2.38	0.151	<0.0001	0.000
b*	18.26	19.22	19.04	18.36	18.32	0.176	0.145	0.091
Regression equation								***R*²**
L* = 43.6813 – 0.5116*x*								0.46
a* = −0.6481 + 0.7922*x*								0.93

539 SEM, standard error of mean; *x*, extract concentration; R^2, coefficient of determination.

Table 5 - β-Carotene and xanthophyll content in experimental diets and yolk from Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract

Parameter	**Extract concentration (g/kg diet)**				
	0	**1**	**2**	**3**	**4**
β-Carotene (mg/kg)[1)]					
Diet	4.16	5.66	13.76	17.07	20.36
Lyophilized egg yolk	14.70	23.04	36.37	49.50	66.68
Xanthophyll (mg/kg)[1)]					
Diet	12.54	13.28	21.64	26.72	35.19
Lyophilized egg yolk	16.77	22.32	45.41	54.74	60.79

[1)] Determined on 2 g samples.

542

Table 6 - Serum profile of male and female Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract

Parameter	**Sex**	**Extract concentration (g/kg diet)**					**SEM**	***P*-value**	
		0	**1**	**2**	**3**	**4**		**Linear**	**Quadratic**
Albumin	Female	1.97	2.05	1.91	1.86	2.01	0.142	0.145	0.186
(g/dL)	Male	1.83	2.21	2.47	2.11	1.66	0.303	<0.0001	<0.0001
Total protein	Female	4.55	3.49	3.66	3.46	3.81	0.527	<0.0001	0.002
(g/dL)	Male	3.20	2.81	2.63	2.87	3.03	0.538	0.019	0.022
Cholesterol	Female	102.02	126.08	104.90	78.19	98.94	16.76	0.917	0.505
(mg/dL)	Male	276.28	276.13	284.91	294.21	323.71	56.73	0.813	0.455
Triglycerides	Female	599.75	622.55	416.73	400.00	406.44	61.87	0.011	0.172
(mg/dL)	Male	156.38	140.60	135.50	110.16	162.08	39.50	0.052	0.062

Regression equations	R^2	**Vertex *x***
Male albumin = $1.8148 + 0.6114x - 0.1632x^2$	0.48	1.87
Female triglycerides = $606.4077 - 60.9174x$	0.65	-

543 SEM, standard error of mean; *x*, extract concentration; R^2, coefficient of determination.

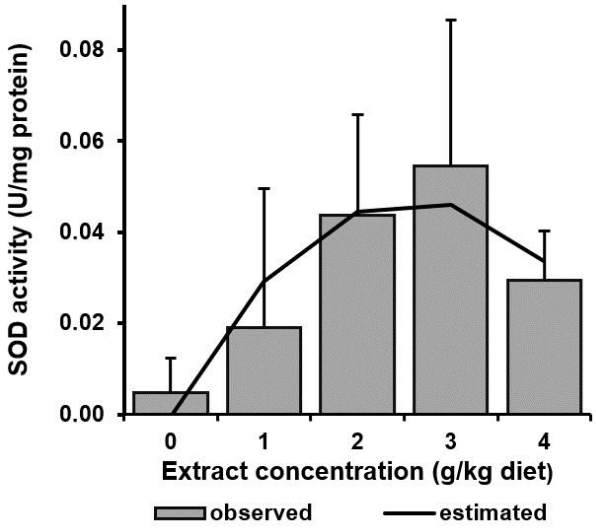
544

Table 7 - Serum antioxidant activity and lipid peroxidation in Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract

Parameter	**Extract concentration (g/kg diet)**					**SEM**	***P*-value**	
	0	**1**	**2**	**3**	**4**		**Linear**	**Quadratic**
Antioxidant activity								
ABTS	33.803	35.055	45.266	50.039	56.182	6.127	0.253	0.748
DPPH	50.626	48.000	48.308	49.579	49.087	1.60	0.038	0.048
Lipid peroxidation (conjugated diene levels)								
V_{max}	0.011	0.005	0.007	0.006	0.007	0.001	0.157	0.224
T_{max}	221.219	251.400	272.997	221.652	278.012	14.61	0.613	0.787
OD_{max}	0.027	0.017	0.023	0.020	0.015	0.002	0.628	0.932

545 SEM, standard error of mean; V_{max}, maximum rate of conjugated diene (CD) production (nmol CD/min); T_{max},
546 time to reach V_{max} (min); OD_{max}, optical density at the maximum CD accumulation.

547

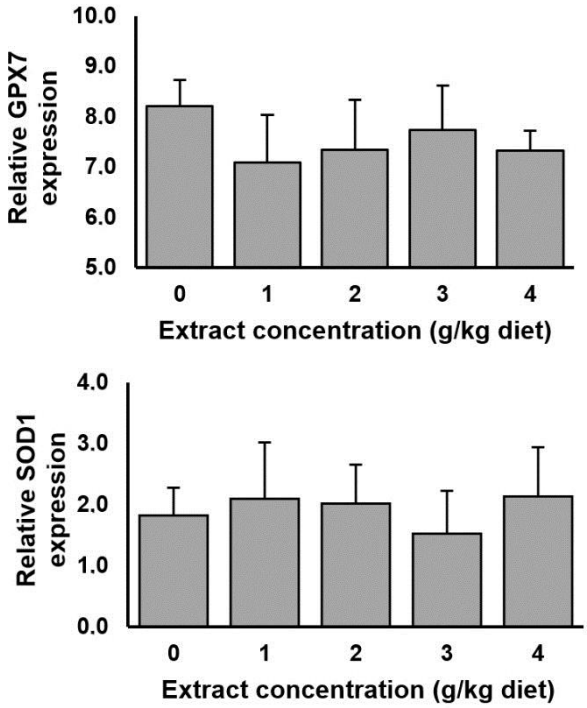


548

Figure 1 - Superoxide dismutase (SOD) activity in the liver of Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract.

549 Results are presented as mean and standard deviation. Regression equation: SOD activity =
550 $-0.00049 + 0.0365x - 0.0070x^2$, where x is the extract concentration; coefficient of
551 determination = 0.38; standard error of mean = 0.022; vertex x = 2.61 g extract/kg diet.

552



553

Figure 2 - Relative expression of glutathione peroxidase (GPX7) and superoxide dismutase (SOD1) genes in the vaginal mucosa of Japanese quail (*Coturnix coturnix japonica*) breeders fed diets containing different concentrations of *Tagetes erecta* extract.

554 Results are presented as mean and standard deviation. *GPX7* expression: standard error of
555 mean = 0.352, *P*-value (linear model) = 0.190, *P*-value (quadratic model) = 0.271. *SOD1*
556 expression: standard error of mean = 0.366, *P*-value (linear model) = 0.820, *P*-value
557 (quadratic model) = 0.810.

IV. DIETARY SUPPLEMENTATION OF JAPANESE QUAIL BREEDERS WITH *TAGETES ERECTA* FLOWER EXTRACT INFLUENCES EGG FERTILITY, INCUBATION PARAMETERS, AND PROGENY PERFORMANCE

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Abstract

Carotenoids are used in layer poultry diets to enhance yolk color and reproductive performance. These compounds are transferred to the yolk, promoting beneficial effects on the oxidative profile of progeny. Although quail are excellent models for reproductive studies, there are few reports on the effects of maternal diet on progeny performance. This study aimed to investigate the effects of supplementation of Japanese quail (*Coturnix coturnix japonica*) breeders with *Tagetes erecta* flower extract on productive performance, egg quality, chick quality, antioxidant capacity, and lipid peroxidation. A total of 480 quail breeders were distributed in a completely randomized design, with 5 extract concentrations (0, 1, 2, 3, and 4 g/kg diet) and 12 replications. Each experimental unit consisted of six females and two males. Productive performance was assessed by evaluating eggs collected during four consecutive days from 12- and 25-wk-old broilers. The fertility of eggs from 12-wk-old quail was 100%, and extract levels had a quadratic effect on the fertility of eggs from 25-wk-old quail. Weight at hatching decreased linearly with extract concentration, but chick quality (Pasgar score) was not affected. The antioxidant capacity of the yolk was not influenced by extract levels, but pre-incubation storage at 20 °C for 8 d increased malondialdehyde levels in egg yolk, embryo yolk sac, and chick liver. Liver superoxide dismutase activity in 3-d-old quail was not influenced by extract concentrations in maternal diets. Thus, the dietary extract concentrations used were not sufficient to enhance the antioxidant profile of the yolk sac or chick liver. In progeny of 12-wk-old breeders, extract levels had a quadratic effect on feed intake from 1 to 14 d of age (maximum at 2.02 g/kg diet) and body weight (maximum at 2.02 g/kg diet). In progeny of 25-wk-old breeders, extract levels exerted quadratic effects on feed intake (maximum at 2.09 g/kg diet) and feed conversion ratio (maximum at 2.18 g/kg diet) from 1 to 14 d and a linear effect on feed intake from 15 to 28 d. *T. erecta* flower extract improved the nutrition status of older breeders, and dietary supplementation is a promising strategy to increase egg fertility.

596 **Keywords**: *Tagetes* extract, xanthophyll, superoxide dismutase, antioxidant, embryo.

597

Introduction

Grower and layer diets for poultry breeders are routinely supplemented with antioxidant compounds. Antioxidant supplementation is aimed at improving productive performance and the oxidative stability of poultry products (Yesilbag et al. 2013). Several natural compounds exhibit antioxidant properties, including vitamin E, vitamin C (ascorbic acid), polyphenols, and carotenoids.

Carotenoids are yellow, orange, and red pigments commonly found in plants. When added to poultry diets, carotenoids enhance the color of meat (Bonamigo, 2017) and eggs (Sandeski, 2016). But the benefits of carotenoid use in animal feed are not limited to color enhancement; these natural pigments exert phytochemical, nutraceutical, and antioxidant effects by stabilizing free radicals and transferring unpaired electrons to other antioxidant substances (Surai 2012). Flowers of marigold (*Tagetes erecta*) are particularly rich in xanthophylls, oxygenated carotenoids with beneficial health effects associated with their high antioxidant and radical scavenging capacity. The major xanthophyll in *T. erecta* extract is lutein.

A high carotenoid content in the yolk sac has been associated with increased embryo viability and subsequent hatchability (Karadas et al. 2005). The yolk sac contains large amounts of lipids and fat-soluble substances, requiring antioxidant protection during incubation. It is known that xanthophylls are the major carotenoids in egg yolk and are efficiently transferred from the yolk sac to different organs and tissues of the developing embryo. Thus, xanthophylls may help protect *in ovo* chicks against oxidative damage, particularly during stressful events such as hatching and the first days of life.

Bird embryo tissues contain a high proportion of polyunsaturated fatty acids. Newly hatched chicks possess a variety of antioxidant defenses, including natural antioxidants (e.g., vitamin E, carotenoids, glutathione, and ascorbic acid), antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase, and catalase), and enzyme cofactors (e.g., selenium, zinc, manganese, and iron). Whereas vitamin E, carotenoids, and metals are derived from the

maternal diet and transferred to the embryo through the yolk sac, other antioxidant substances are synthesized by embryonic tissues (Surai 2000). A major function of the antioxidant defense system is to mitigate the damages caused by free radicals and inhibit lipid peroxidation. These findings indicate that maternal supplementation with carotenoids or xanthophylls may be a practical and effective strategy to enhance the antioxidant defenses and optimize the performance of both breeders and their progeny.

Despite the various studies on antioxidant supplementation of broilers, layers, and breeders, there are still many gaps in our understanding of the physiological effects of different antioxidant sources on adult and developing birds. In this study, we aimed to investigate the effects of *T. erecta* extract supplementation of Japanese quail (*Coturnix coturnix japonica*) breeders on incubation performance, hatchability, chick quality, antioxidant capacity, and lipid peroxidation.

Material and methods

All experimental procedures were approved by the Animal Ethics Committee of the State University of Maringá, Paraná, Brazil (protocol no. 3486021017).

2.1. Animals, experimental design, and diets

Male and female Japanese quail breeders were randomly allocated into groups of eight birds (six females and two males) matched for body weight (females, 146.33 g; males, 121.00 g) and egg production (91.16 %). The animals were housed in galvanized wire cages (25 × 39 cm) with a nipple drinker at the top and a trough feeder at the front and maintained under a 17 h light (natural + artificial)/7 h dark photoperiod with *ad libitum* access to feed and water. Female and male birds in the same cage were fed the same diet. The maximum (29.72 °C) and minimum (16.57 °C) ambient temperature and maximum (60.76%) and minimum (40.46%) relative humidity were measured daily using a portable thermohygrometer.

A total of 480 layer-type quail breeders aged 12 wk were distributed in a completely randomized design, with five treatments (0, 1, 2, 3, and 4 g *T. erecta* extract/kg diet) and 12 replications. Diets were based on corn and soybean meal (Table 1) and met the requirements of quail breeders, according to Rostagno et al. (2017). To obtain five isoenergetic and isonitrogenous diets, we replaced adequate amounts of inert (kaolin) with equal amounts of *T. erecta* extract. The commercial extract, produced by solvent extraction of *T. erecta* petals, contains a guaranteed level of 20 mg xanthophyll/kg. Fresh feed was prepared and homogenized every 15 d and stored in dark bags in a dry, dark environment.

Eggs produced after 15 d of dietary treatment were collected and evaluated for incubation performance, hatchability, chick quality, and progeny performance.

2.2. Incubation performance and chick quality

Incubation performance was evaluated in eggs from birds aged 12 and 25 wk. Eggs were collected from each experimental unit (cage) for 4 d and stored in a refrigerated room at 20 °C during the collection period. Then, eggs were incubated in a vertical incubator with a total capacity of 3,978 quail eggs (Labo 13, Petersime, Belgium) at 60% relative humidity and 37.6 °C with automatic turning every hour. After 14.5 d of incubation (348 h), the eggs were transferred to a hatcher (Labo 9, Petersime) and kept at 70% relative humidity and 37.0 °C until hatched (total of 408 h of incubation).

The quality of newly hatched chicks (50/treatment/maternal age) was assessed by analyzing their body weight and Pasgar score. For Pasgar score analysis, it was considered that all chicks start with a score of 10 and lose 1 point for each defect observed for reflex (reaction of chicks to a stimulus), navel (healing status), legs and beak (lesions), and belly characteristics (degree of yolk absorption). Unhatched eggs were opened and analyzed for quantification of infertile eggs and dead embryos. Embryo mortality was classified according to the stage of embryonic development into the following categories: early and mid mortality

(1 to 11 d of incubation), late mortality (more than 12 d of incubation), and mortality at pipping (pipped unhatched eggs). The number of eggs in each category was used to estimate the hatching percentage (determined in relation to the total number of fertile eggs), fertility, infertility, total, early, mid, late, and pipping mortality.

2.3. Progeny performance

Progeny performance was analyzed from 1 to 28 d of age in chicks hatched from 12- and 25-wk-old breeders. One-day-old chicks were distributed according to each treatment in 1 m^2 boxes equipped with a chick drinker and a tubular feeder and maintained in a controlled environment under 24 h artificial light. All chicks were fed the same starter (1 to 14 d) and grower (15 to 28 d) diets, formulated according to Rostagno et al. (2017).

The design was completely randomized, with 5 treatments, 4 replications, and 50 birds per experimental unit. Chicks and feed were weighed weekly to determine the feed intake and body weight gain at 7, 14, 21, and 28 d of age. Productive performance was evaluated in terms of mean feed intake (g), body weight (g), weight gain (g), and feed conversion ratio (g feed/g meat) in the starter and grower phases.

2.4. Egg storage and sample collection for lipid peroxidation analysis and weight determinations

The effects of egg storage before incubation on antioxidant activity and lipid peroxidation in yolk and liver tissues were assessed in 200 eggs per experimental diet, totaling 500 eggs per storage period (0 and 8 d). Eggs were weighed and stored in a refrigerated room at 20 °C for 8 d. On the day of incubation, stored and non-stored eggs were weighed and incubated under the conditions described in Section 2.2. Developing embryos were evaluated at 7, 11, and 15 d of incubation, and chicks were evaluated at hatching (17 d of incubation) and 3 d post-hatch.

2.5. Egg, embryo, and yolk weights

Egg weight loss during incubation was determined in the same eggs (n = 20 per treatment per storage period) used for analysis of antioxidant activity and lipid peroxidation. First, the weight of empty egg trays was determined. Then, the eggs were trayed, weighed, incubated, and weighed again on the day of transfer to the hatcher (15 d after incubation). The mean weight of eggs was calculated on each weighing occasion, and weight loss (%) was expressed in relation to the initial egg weight. Embryo weights were determined after 15 d of incubation, and chick weights at hatching and 3 d post-hatch (n = 20 eggs per experimental diet per storage period). Chicks were reared under the conditions described in Section 2.3 until 3 d post-hatch. Embryos and chicks were sacrificed by cervical dislocation. Yolk from eggs before and during incubation, residual yolk, and liver specimens were dissected, and weighted to obtain the relative organ weights. The yolk and residual yolk contend were separated from the vitelline membranes before pooled. Samples were pooled per treatment, homogenized, aliquoted into four fractions in 1.5 mL microtubes, frozen in liquid nitrogen, and stored at −80 °C until freeze-drying. Before this step, microtubes were removed from the freezer and placed in liquid nitrogen. The frozen material was freeze-dried for 72 h and stored at −80 °C until analysis.

2.6. Sample preparation for determination of lipid peroxidation, antioxidant activity, and superoxide dismutase (SOD) activity

Yolk, yolk sac, and liver samples were evaluated for lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) method. Fresh eggs were also evaluated for antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Liver specimens from 1-d-old chicks were evaluated for SOD activity. Determinations were

performed in a pool of three samples per experimental diet. Samples were ground in liquid nitrogen and homogenized before analysis.

2.7. Determination of antioxidant activity

Antioxidant activity was determined in fresh egg yolk by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), following the method described by (Li et al. 2009), with modifications. Serum aliquots (150 µL) were mixed with 2.85 mL of 60 µM DPPH solution and incubated in the dark for 30 min. Absorbance was read at 515 nm, and antioxidant activity (%) was calculated using Eq. (2):

$$DPPH = (1 - Abs/Abs_0) \times 100 \quad (2)$$

where Abs is the sample absorbance at 30 min and Abs_0 is the sample absorbance at time 0. The result was expressed as half maximal inhibitory concentration (IC_{50}, the lowest concentration required to inhibit DPPH activity by 50%).

2.8. Determination of lipid peroxidation

Solutions (0.500 µL) of 1,3,3-tetramethoxypropane in 50% methanol at different concentrations (0, 10, 20, 30, 40, 50, and 60 mM) were mixed with 2.0 mL of thiobarbituric acid (TBA) solution in a conical centrifuge tube and incubated in a water bath at 100 °C for 15 min. After resting for 5 min, the solutions were analyzed spectrophotometrically at 532 nm. Absorbance results were used to construct a standard curve ($R^2 \geq 0.99$) for TBARS determination.

Lipid peroxidation was determined by the TBARS method, adapted from (Vyncke 1970). The extract solution was prepared by adding 0.1 g of freeze-dried sample to 1.9 mL of 15% trichloroacetic acid (TCA). Then, 1 mL of sample extract was homogenized with 1.0 mL of a solution containing 1% TBA, 15% TCA, and 0.06% HCl, incubated in a boiling water bath for 15 min, and left to rest for 5 min. As a control procedure, the absorbance of TBA and

753 TCA solutions was measured at 352 nm to verify the calibration of the spectrophotometer.
754 Sample absorbance was measured at 532 nm on a spectrophotometer. TBARS results were
755 determined against the standard curve of malondialdehyde (MDA) and are presented in mg
756 MDA/kg sample.
757
758 2.9. Determination of SOD activity
759 SOD activity was measured in a pool of five chick liver samples per treatment. The
760 reaction is based on the sample's ability to inhibit the autoxidation of pyrogallol in alkaline
761 medium at 420 nm (Marklund and Marklund, 1974). One unit (U) of SOD was defined as the
762 amount of enzyme needed to inhibit the reaction, and results are expressed in U/mg protein.
763
764 2.10. Statistical analysis
765 For investigating the effects of maternal diet and egg storage on yolk sac and liver
766 antioxidant activity and lipid peroxidation during and after incubation, each pool of four eggs
767 was treated as an experimental unit (n = 5). For analyses of weight loss during incubation and
768 relative organ weights, each egg, embryo, or chick was considered an experimental unit (n =
769 20). For analysis of Pasgar scores, each chick was treated as an experimental unit (n = 50).
770 Treatment effects were assessed by analysis of variance between storage periods, and
771 regression analysis of extract concentrations, incubation periods, and their interactions was
772 performed. These statistical analyses were conducted using SAS software version 2009. The
773 level of significance was set at $P < 0.05$.
774 Differences in incubation performance were considered significant at $P < 0.10$. Fertility,
775 hatching, and embryonic mortality were modeled using the GENMOD procedure of SAS with
776 a binomial probability distribution and a logit link function ($\text{LOGIT} = \frac{e^{\beta}}{1 + e^{\beta}}$). When analyzing
777 incubation performance data, we randomly selected six experimental units per incubation

778 period to reduce the number of repeated measurements per time point. Probability estimates
779 of incubation performance were transformed to percentage values by multiplying by 100.
780
781 **Results**
782 Egg fertility probability was influenced by maternal age and diet (Table 2). The fertility of
783 eggs from 12-wk-old breeders was estimated at 100%, and diet was found to not influence the
784 parameter. For 25-wk-old quail, diet had a quadratic effect ($P = 0.034$) on egg fertility (Fig.
785 1). Infertility was estimated as a percentage of fertility (100% – fertility) and had a negative
786 quadratic relationship with extract concentration (Fig. 1).
787 Maternal age influenced the hatching probability of fertile eggs ($P = 0.017$), which was
788 higher in quail aged 25 wk (95.06%) than in quail aged 12 wk (90.16%). Total mortality was
789 determined as a percentage of hatchability (100% – hatchability). Early, mid, and late
790 mortality and mortality at pipping were determined in relation to total mortality and assessed
791 by probability analysis. Early and mid mortality was 5.45% for 12-wk-old quail and 25.93%
792 for 25-wk-old quail. There was a higher percentage of late embryonic mortality and mortality
793 at pipping. Eggs from 12-wk-old quail had a higher late mortality (94.55%) than eggs from
794 25-wk-old quail (74.07%).
795 Chick quality was assessed by weight at hatching, and a negative linear relationship was
796 observed between weight and extract concentration ($P < 0.05$). Chick weight was higher
797 among progeny of 25-wk-old breeders. Morphological variables used to calculate the Pasgar
798 score were not influenced ($P > 0.05$) by maternal age or diet (Table 3).
799 Fig. 2 shows the plot of egg weight loss as a function of incubation period. Eggs stored at
800 20 °C for 8 d prior to incubation lost 2.6% of their initial weight. Egg weight loss had a
801 quadratic relationship with incubation period but was not influenced by maternal diet. Yolk
802 weight was influenced ($P < 0.05$) by storage, incubation period, maternal diet, and the
803 interaction incubation period × maternal diet (linear effect, Fig. 3). Yolk weight decreased

804 with increasing incubation period and extract concentration in maternal diets. Interaction
805 effects ($P < 0.05$) between maternal diet and egg storage were also observed. Non-stored eggs
806 had a higher yolk weight at the end of incubation than stored eggs.

807 Maternal diet had a cubic effect ($P = 0.04$) and incubation period exerted a quadratic effect
808 ($P < 0.0001$) on embryo weight (Fig. 4). Egg storage did not influence ($P < 0.05$) embryo or
809 chick weight. Chick weight increased during incubation, with a marked increase between 11
810 and 15 d and a 3.9-fold increase between 11 and 17 d. At 15 d of incubation, liver weight did
811 not differ ($P > 0.05$) from that at 1 d of incubation, regardless of egg storage or maternal diet.
812 Liver weight only differed significantly between 15 d (103 mg) and 17 d (227 mg) of
813 incubation.

814 The antioxidant capacity of egg yolk, as assessed by the DPPH method, was not influenced
815 ($P > 0.05$) by maternal diet (Fig. 5). We assessed lipid oxidation in egg yolk, embryo yolk sac
816 and liver at 15 d of incubation, yolk sac and liver of 1-d-old chicks, and liver of 3-d-old
817 chicks (Table 4). Egg storage increased MDA levels in all tissues. Maternal diet did not affect
818 ($P > 0.05$) lipid oxidation.

819 Fig. 6 shows the liver SOD activity of 1-d-old chicks from quail supplemented with *T.*
820 *erecta* flower extract. Extract concentration did not influence this parameter.

821 Progeny performance was not affected ($P > 0.05$) by maternal age (12 or 25 wk). For
822 progeny of 12-wk-old breeders, extract concentration had a quadratic effect ($P < 0.05$) on feed
823 intake from 1 to 14 d. The highest feed intake was estimated to be achieved by maternal
824 supplementation with 2.48 g/kg extract. Maternal diet also had a quadratic effect on body
825 weight at 28 d. An extract concentration of 2.02 g/kg was estimated to afford the highest
826 chick body weight at 28 d (Table 5).

827 The feed intake and feed conversion ratio (1–14 d) of progeny of 25-wk-old quail were
828 quadratically influenced ($P < 0.05$) by maternal diet (Table 6). Regression analysis showed

that the highest feed intake and feed conversation ratio could be achieved by supplementing maternal diets with 2.09 and 2.18 g/kg extract, respectively. In the grower phase (15–28 d), feed intake increased linearly ($P < 0.05$) with extract concentration in the maternal diet.

Discussion

Maternal age greatly influenced the probability of fertility, infertility, hatchability, and total mortality. Significant interaction effects of maternal age × maternal diet were observed only on fertility and infertility. Fertility and hatchability of fertile eggs are important indicators of the reproductive capacity of poultry breeders. Both parameters are influenced by numerous factors, including maternal age and diet as well as incubation conditions.

In hens, young breeders generally produce smaller eggs than breeders at the peak laying phase, leading to low egg yield and chick weight at hatching. These effects, associated with a lower capacity to transfer lipids to the egg yolk, can compromise embryonic viability in late stages of development and reduce hatchability (Benton and Brake, 1996; Fasenko, 2003). According to (Rocha et al. 2008), the absolute and relative weights of the yolk sac are influenced by egg size. Smaller eggs produced by young breeders have a lower number of pores, thicker membrane and cuticle, denser albumen, and smaller nutrient reserves, leading to the formation of small chicks. As bird age increases, eggshell, cuticle, and membranes become thinner, increasing the concentration of yolk nutrients and leading to the formation of larger chicks (Mcloughlin and Gous, 2000).

Poultry breeder nutrition also influences egg composition, embryonic development (Wilson 1997), and chick weight, vigor, and immune status (Kenny and Kemp, 2005). Embryonic development is dependent on environmental conditions and the nutrients available in the egg.

The carotenoid content of eggs is positively associated with embryonic viability and hatchability (Karadas et al. 2005). Carotenoids, of which xanthophylls are important

representatives, play a major antioxidant role (Böhm et al. 1997). In birds, dietary pigments such as carotenes are absorbed from the intestinal lumen and transported together with lipids into cells via lipoproteins present in the cell membrane. These pigments can accumulate in lipid-rich tissues, such as egg yolk (Faehnrich et al. 2016). When added to breeder diets, carotenoids can protect embryonic tissues and yolk nutrients rich in polyunsaturated fatty acids from oxidation during storage and incubation. According to Rutz et al. (2005), if reproductive tissues do not have effective antioxidant mechanisms, the action of free radicals may decrease the fertility of breeders.

Alay and Karadas , (2016) investigated the effects of supplementation of quail breeder diets based on wheat bran with 10 mg/kg of five natural pigments, including *T. erecta* extract. The authors did not observe significant effects on egg fertility; hatchability, on the other hand, was higher with apo-ester (yellow carotenoid) supplementation, which produced similar effects to *T. erecta* extract supplementation.

Another factor that can alter egg composition and lead to embryonic death is egg storage prior to incubation. Storage may induce the formation of free radicals and stimulate lipid peroxidation in blastoderm cells. As a result, the rate of oxygen consumption during incubation is enhanced, compromising embryonic development (Surai 1999). During storage and incubation, eggs lose water by diffusion through eggshell pores, leading to weight loss. The intensity of weight loss in fertile eggs depends on the temperature and humidity conditions of storage (Véras et al., 2000) and incubation (Decuypere et al., 2003). Egg weight loss provides important information on gas exchange in embryos (Paganelli et al., 1978; Rahn et al., 1979) and is correlated with embryonic metabolism and development (RAHN and AR 1980). Our results are in line with those of (Scott and Silversidest 2000), who observed a progressive loss of egg quality and weight over 10 d of evaluation. In this way, Soliman et al. (1994) observed that Japanese quail eggs lose 11.3% of their initial weight from 1 to 15 d of

incubation. In an experiment using 0 and 6 ppm canthaxanthin (Carophyll red), Rocha (2011) assessed the effects of storage period and breeder supplementation and found no effects on egg weight loss during incubation. Also in quail, but studding meat-type subspecie, (Moraes et al. 2009) also had no investigated the effects of low storage temperatures (7.5 ± 1 °C) on incubation and egg parameters and found no correlation between storage period and weight loss during incubation or storage period and weight at hatching.

In the present study, yolk and embryo weights were influenced by maternal supplementation with *T. erecta* flower extract and incubation period. Pre-incubation egg storage did not affect these variables, as also observed by (Pedroso et al. 2006) in studying the incubation of fertile eggs of 19-wk-old Japanese quail. The authors reported that embryo and embryonic appendages were not influenced by storage period (0, 72, and 144 h).

Embryo weight at 18 d of incubation was also not influenced by storage period × maternal canthaxanthin supplementation in the study of Rocha (2011). However, a 7-d storage period reduced ($P \leq 0.01$) embryo weight at 18 d compared with a 3-d storage period. The relative weight of newly hatched chicks from eggs stored for 2 d was lower than that of chicks from non-stored eggs (Ferreira, 2006). In contrast, (Tanure et al. 2009) found that 3 d of storage reduced chick weight compared with 7 d of storage. The poorer embryonic development observed in the shorter storage period might have been related to lower albumen liquefaction, resulting in lower oxygen and nutrient supply to the embryo and lower body tissue deposition.

According to Fernandes (2017), the smaller the amount of an antioxidant needed to reduce the initial amount of DPPH to 50%, the greater its antioxidant capacity. The concentrations of *T. erecta* flower extract used in maternal diets were not sufficient to promote a greater deposition of carotenoids in the yolk or enhance antioxidant capacity.

Results in this study from stored eggs during 8 days before incubation in a room with 20º C promoted lipid oxidation in eggs, yolk, and embryo and chick liver. MDA, a secondary

oxidation product, is used as an indicator of lipid oxidation, which is known to increase during storage (Pappas et al., 2005; Mohiti-Asli et al., 2008; Ren et al., 2013). Rezaei et al. (2019) reported that storage increased MDA levels in egg yolk, as also found in the present study. (Mohiti-Asli et al. 2008), Hayat et al. (2010), and Wang et al. (2017) highlighted that storage temperature and maternal diet can significantly affect egg quality and oxidative stability. Radwan et al. (2008) associated the TBARS content in newly laid eggs with TBARS levels in maternal diets; according to the authors, such compounds are transferred to the yolk. On the other hand, (Skřivan et al. 2016) reported that the carotenoids lutein and zeaxanthin from *T. erecta* flower extract can be deposited in yolk.

In studies in laying hen supplemented with lutein (250 mg/kg), Englmaierpvá et al. (2013) observed a significant increase in yolk lutein concentration and an improvement in the oxidative stability of fresh eggs and eggs stored for 28 days. (Alay and Karadas 2016) found that Japanese quail fed a diet containing 2 g/kg *T. erecta* flower extract (10.6 mg/kg lutein) produced yolks containing 3.11 mg/100 g lutein and 0.30 mg/100 g zeaxanthin. In our results, dietary levels of *T. erecta* flower extract might have been insufficient to improve the oxidative stability of eggs, yolk sac, and chick liver.

The effects of carotenoids on breeder´s diets and progenies' effects are described in some avian species. Studies in broilers of Gao et al. (2013) showed that diets of breeder supplemented with xanthophylls (40% lutein and 60% zeaxanthin, 0 or 40 mg xanthophylls/kg) changed the antioxidant capacity and lipid peroxidation of the chicks. Results of this study demonstrated that liver MDA levels of chicks from supplemented parents were lower at 0 and 7 d of age. Chick supplementation reduced liver MDA concentrations at 21 d of age. Xanthophyll supplementation, however, did not influence SOD activity (up to 21 d).

In general, *T. erecta* flower extract contains 12 g/kg xanthophylls, 80–90% of which is lutein (Moura et al., 2011; Ševel et al., 2014). Rosa et al. (2012) found that carotenoids play a major role in the antioxidant and immune systems of newly hatched chicks, which is why maternal supplementation with carotenoids is a promising strategy to improve progeny performance.

(Karadas et al. 2006) supplemented maternal diets with tomato powder and *T. erecta* flower extract and observed a positive influence on the antioxidant system of growing quail. Lycopene and lutein concentrations in the egg yolk were high, leading to high concentrations of coenzyme Q_{10} (ubiquinone) in the liver of newly hatched chicks. The authors stated that the increase in carotenoid and liver coenzyme Q_{10} contents positively contributed to the immunocompetence of growing chicks. Coenzyme Q_{10} is a lipophilic antioxidant found in the mitochondria of all cells and is involved in the transfer of electrons in the oxidative respiratory chain and adenosine triphosphate production (Geng et al., 2004). Studies with broilers demonstrated a relationship between feed conversion ratio and mitochondrial function (Iqbal et al., 2004; Bottje and Carstens, 2009; Kalantar et al., 2019), as also observed in the present study.

Lutein is a potent antioxidant with immunomodulatory properties; the carotenoid acts mainly on T lymphocytes (Chew, 1993). Production of T lymphocytes is mainly carried out by cells associated with the small intestine mucosa (van der Heijden et al. 1987). The close relationship between animal immune status and nutrition is made evident by the action of lutein on the immune system, consequently improving gastrointestinal tract health, digestion, and nutrient absorption; such effects enhance animal performance (Alarça, 2012).

Surai and Speake (1998) found that dietary supplementation of carotenoids increased deposition on egg yolk and chick organs. Yolk carotenoid concentrations can be easily increased by supplementation of breeder diets. In line with these results, Koutsos et al. (2003)

demonstrated that the carotenoid content of maternal diets influences the concentration of these compounds in progeny tissues up to 28 d of age.

The literature is clear in that breeder age influences the quality, composition, and size of eggs. As maternal age increases, egg productivity decreases but yolk and albumen size increase (Rocha et al., 2008). In this study, maternal age did not influence chick performance, as assessed by the Pasgar score. Day-old chicks were considered to be of good quality, with an average score of 9 (Pas Reform, 2008). This result corroborates those of Santos et al. (2015), who found that the age of European quail breeders (85, 140 and 270 d) did not influence progeny performance.

Conclusions

The dietary concentrations of *T. erecta* flower extract generally used for enhancing egg yolk color were not sufficient to improve breeder productive performance or the antioxidant potential and performance of progeny. However, the beneficial effects of *T. erecta* flower extract supplementation on egg fertility showed that this supplement plays an important role in the nutrition of older breeders and represents an important strategy to improve fertility in quail.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figures and Tables

Table 1 - Ingredient composition, calculated nutrient content, and energy value of basal diets fed to Japanese quail (*Coturnix coturnix japonica)* breeders and chicks at the starter and grower phase

Ingredient, %	Breeder	Chick	
	Layer	Starter (1–14 d)	Grower (15–28 d)
Corn	61.30	54.03	58.55
Soybean meal	28.88	38.90	35.94
Limestone	6.78	1.05	0.89
Soybean oil	0.21	2.23	1.28
Dicalcium phosphate	1.11	2.20	1.75
Vitamin–mineral premix[1,2]	0.40	1.00	1.00
Sodium chloride	0.27	0.43	0.46
DL-Methionine (98.5%)	0.25	0.15	0.12
L-Lysine (76.5%)	0.38	0.01	0.01
Inert[3]	0.40	-	-
Calculated composition			
Crude protein (%)	18.92	22.00	21.00
Metabolizable energy (kcal/kg)	2,800	2,900	2,900
Calcium (%)	2.99	1.092	0.91
Available phosphorus (%)	0.309	0.513	0.43
Sodium (%)	0.147	0.205	0.21
Digestible lysine (%)	1.149	1.095	1.03
Digestible methionine (%)	0.517	0.447	0.41
Digestible methionine + cysteine (%)	0.942	0.744	0.69

[1] Provided per kilogram of product in breeder diets: 2,500,000 IU vitamin A, 500,000 IU vitamin D3, 5,000 IU vitamin E, 625 mg vitamin B1, 1,500 mg vitamin B2, 1,250 mg vitamin B6, 5,000 IU vitamin B12, 750 mg vitamin K3, 3,000 mg calcium pantothenate, 6,000 mg niacin, 250 mg folic acid, 50.0 mg biotin, 75 g choline, 1,000 mg antioxidant (butylated hydroxytoluene), 13 g zinc, 13 g iron, 15 g manganese, 3,000 mg copper, 50 mg cobalt, 250 mg iodine, and 63 mg selenium.

[2] Provided per kilogram of product in chick diets: 931,000 IU vitamin A, 189,000 IU vitamin D3, 1,500 IU vitamin E, 156 mg vitamin K3, 150 mg vitamin B1, 500 mg vitamin B2, 310 mg vitamin B6, 1,200 IU vitamin B12, 1,250 mg calcium pantothenate, 3,000 mg niacin, 75 mg folic acid, 4 mg biotin, 45 g choline, 1,500 mg antioxidant (butylated hydroxytoluene), 5 g zinc, 5 g iron, 6.05 g manganese, 600 mg copper, 77.5 mg iodine, 25.7 mg selenium, and 2,200 mg zinc bacitracin.

[3] Kaolin.

1063

Table 2 - Probability of fertility or infertility, hatchability or mortality, early, mid, and late mortality, and mortality at pipping of eggs from 12- and 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrationsof *Tagetes erecta* flower extract.

	Fertility[1]	Infertility	Hatchability	Total mortality	Mortality	
					Early and mid	Late and pipping
Age, wk						
12	100.00	0.00	90.16[b]	9.84	5.45[a]	94.55[b]
25	93.82	6.18	95.06[a]	4.94	25.93[b]	74.07[a]
Extract concentration, g/kg diet						
0	98.19	1.81	94.04	5.96	92.31	7.69
1	95.67	4.33	91.40	8.60	94.74	5.26
2	95.27	4.73	94.14	5.86	92.31	7.69
3	96.96	3.04	93.30	6.70	73.33	26.67
4	98.22	1.78	90.04	9.96	86.36	13.64
P-value						
Age	<0.0001	<0.0001	0.002	0.002	0.010	0.010
Extract concentration (linear effect)	0.045	0.045	0.757	0.757	0.240	0.240
Extract concentration (quadratic effect)	0.026	0.026	0.518	0.518	0.377	0.377
Interaction	0.034	0.034	0.698	0.698	0.471	0.471

1064 [1] Data analyzed using binomial probability distribution and a logit link function.

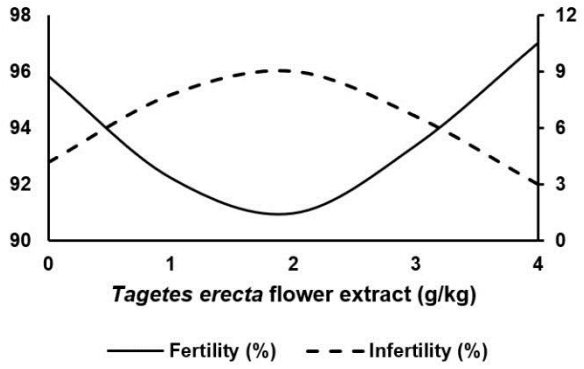
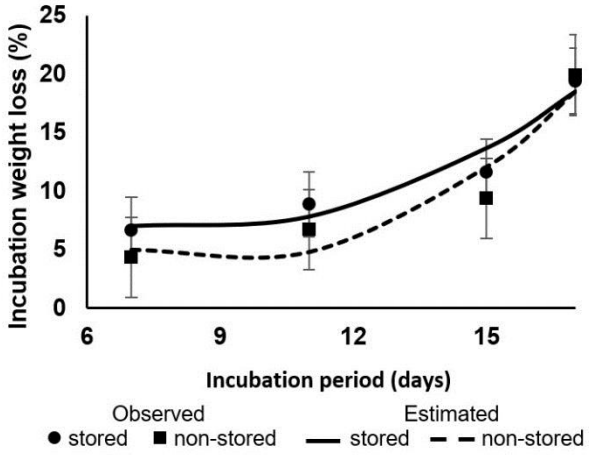


Figure 1 - Effect of *Tagetes erecta* flower extract concentration on the fertility and infertility probability of eggs from 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders.

$\beta = 3.137 - 0.9114C + 0.2495C^2$, where C is the extract concentration; $P = 0.034$.

1068



1069

Figure 2 - Incubation weight loss in stored and non-stored eggs from Japanese quail (*Coturnix coturnix japonic*a) breeders supplemented with *Tagetes erecta* flower extract.

1070 Storage period: 8 d. $R^2 = 0.82$; SEM = 0.283; Stored eggs: $y = 17.8042 – 2.6567t + 0.1587t^2$, where t is the
1071 incubation period, $P < 0.001$; Non-stored eggs: $y = 23.2707 – 4.2421t + 0.2329t^2$, $P < 0.001$.

Table 3 - Weight at hatching and Pasgar score of chicks from 12- and 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract.

Variable	Extract concentration, g/kg diet					SEM	*P*-value	
	0	1	2	3	4		Linear	Quadratic
12-wk-old breeders								
Weight at hatching, g	8.20	7.99	8.06	7.84	7.73	0.207	0.011	0.861
Pasgar score	9.50	9.44	9.50	9.52	9.54	0.656	0.859	0.729
25-wk-old breeders								
Weight at hatching, g	7.85	8.09	7.88	7.85	8.04	0.215	0.743	0.877
Pasgar score	9.50	9.63	9.70	9.53	9.53	0.659	0.300	0.269
Regression equation							R^2	
Weight at hatching (12 wk) = 8.180 − 0.108*C*							0.40	

SEM, standard error of the mean; *C*, extract concentration.

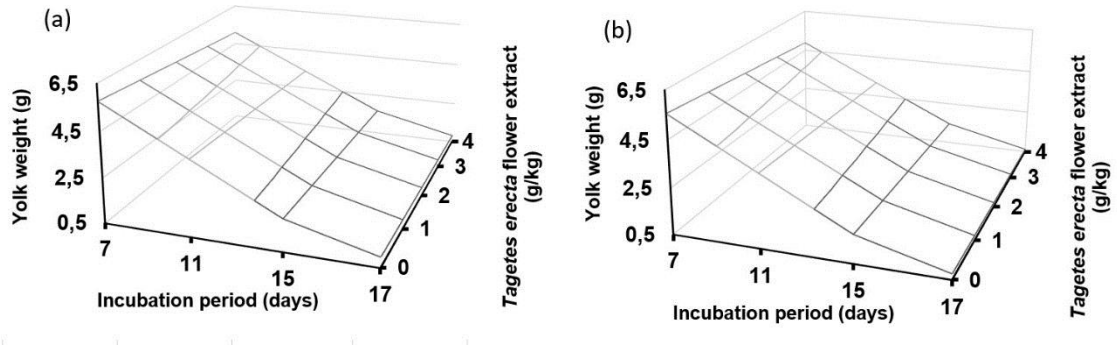
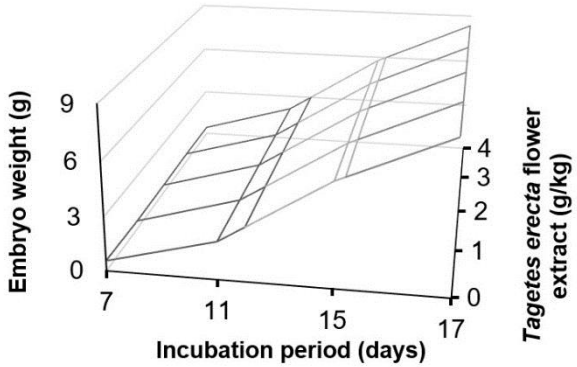


Figure 3 - Yolk sac weight of embryos from eggs stored (a) for 8 d before incubation or (b) non-stored eggs laid by Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of Tagetes erecta flower extract.

R^2 = 0.94; SEM = 0.088; Stored eggs: $y = 9.140 - 0.481t - 0.259C + 0.013tC$, where t is the incubation period and C the extract concentration; Non-stored eggs: $y = 8.931 - 0.481t - 0.259C + 0.013tC$, $P = 0.0001$.



1092

Figure 4 - Embryo weight as a function of egg incubation period and *Tagetes erecta* flower extract concentration in the diet of Japanese quail (*Coturnix coturnix japonic*a) breeders.

1093 Embryo weight: $y = 2.611 - 0.735t + 0.063t^2 - 0.216C + 0.110C^2 - 0.016C^3$, where t is the incubation period and
1094 C the extract concentration; $P = 0.040$; $R^2 = 0.99$; SEM = 0.151.

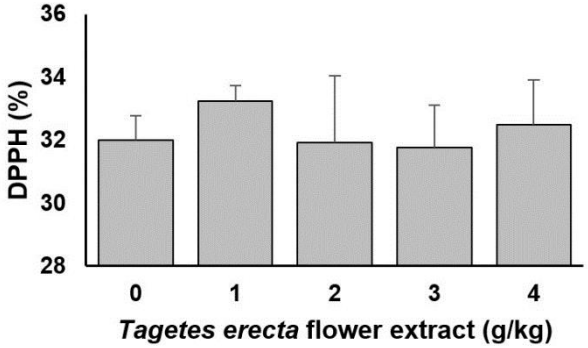


Figure 5 - DPPH radical scavenging activity in the yolk of fresh eggs from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract.

SEM = 0.249, *P* = 0.936.

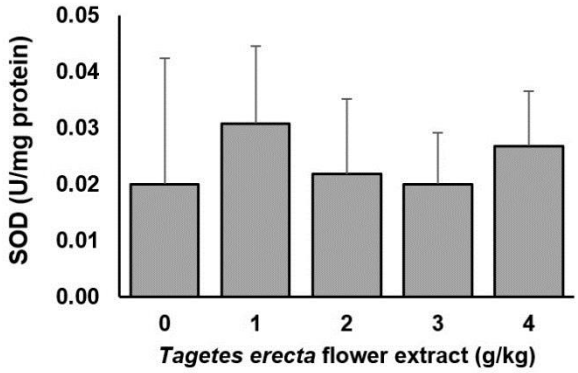


Figure 6- Superoxide dismutase (SOD) activity in the liver of 1-d-old chicks hatched from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract.

SEM = 0.014, *P* = 0.978.

1102

Table 4 - Lipid oxidation in egg yolk, yolk sac, and liver of chicks from Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract.

Variable	MDA (mg/kg freeze-dried sample)					
	Egg yolk	Yolk sac		Liver		
		Embryo (15 d)	Chick (1 d)	Embryo (15 d)	Chick (1 d)	Chick (3 d)
Storage[1]						
No	0.46[b]	1.31[b]	3.75[b]	3.12[b]	3.66[b]	3.88[b]
Yes	0.70[a]	2.06[a]	4.75[a]	4.41[a]	4.87[a]	5.55[a]
Extract concentration, g/kg diet						
0	0.60	1.78	4.49	3.44	4.52	3.23
1	0.64	1.58	4.35	3.82	3.99	5.54
2	0.51	1.47	4.19	3.93	4.50	4.54
3	0.53	1.35	4.04	3.91	4.00	6.13
4	0.49	2.00	3.94	3.92	4.38	4.27
SEM	0.045	0.159	0.175	0.170	0.236	0.362
P-value						
Storage	0.005	0.016	0.003	<0.0001	0.008	0.018
Extract concentration (linear effect)	0.319	0.826	0.265	0.367	0.838	0.133
Extract concentration (quadratic effect)	0.933	0.217	0.954	0.577	0.713	0.062
Interaction	0.888	0.104	0.343	0.384	0.643	0.733

1103 SEM, Standard error of the mean.
1104 [1] At 20 °C for 8 d.

Table 5 - Performance of starter and grower chicks from fertile eggs of 12-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract.

Variable	Extract concentration, g/kg diet					SEM	*P*-value	
	0	1	2	3	4		Linear	Quadratic
Starter, 1 to 14 d								
BW at 14 d, g	53.46	53.45	54.05	54.44	52.76	1.012	0.839	0.131
Feed intake, g	87.54	89.77	94.99	92.60	91.23	3.088	0.136	0.046
BW gain, g	45.31	45.40	45.47	46.65	44.87	0.973	0.846	0.237
FCR, g/g	1.93	1.98	2.09	1.98	2.04	0.076	0.157	0.233
Grower, 15 to 28 d								
BW at 28 d, g	134.21	136.98	136.29	135.37	135.10	1.336	0.949	0.049
Feed intake, g	226.49	235.59	232.51	227.75	223.09	6.864	0.310	0.072
BW gain, g	81.10	82.79	81.83	80.20	81.203	1.208	0.293	0.447
FCR, g/g	2.79	2.85	2.84	2.84	2.75	0.089	0.582	0.140
Regression equations							R^2	Vertex *C*
Feed intake in the starter phase = $87.0686 + 5.2543C - 1.0583C^2$							0.41	2.48
BW at 28 d = $134.6543 + 1.8199C - 0.4505C^2$							0.28	2.02

BW, body weight; FCR, feed conversion ratio; *C*, extract concentration.

Table 6 - Performance of starter and grower chicks from fertile eggs of 25-wk-old Japanese quail (*Coturnix coturnix japonica*) breeders supplemented with different concentrations of *Tagetes erecta* flower extract.

Variable	Extract concentration, g/kg diet					SEM	*P*-value	
	0	1	2	3	4		Linear	Quadratic
Starter, 1 to 14 d								
BW at 14 d, g	47.46	47.72	46.60	47.75	45.91	1.441	0.252	0.549
Feed intake, g	94.32	101.65	111.73	109.25	94.34	7.286	0.686	0.003
BW gain, g	39.50	39.34	38.21	39.57	37.76	1.369	0.201	0.790
FCR, g/g	2.40	2.59	2.92	2.76	2.49	0.199	0.460	0.006
Grower, 15 to 28 d								
BW at 28 d, g	134.72	135.76	136.10	135.59	135.78	1.511	0.483	0.449
Feed intake, g	222.50	228.34	228.65	230.69	235.25	6.951	0.040	0.957
BW gain, g	80.59	80.81	82.09	81.64	81.99	1.144	0.098	0.567
FCR, g/g	2.76	2.83	2.79	2.82	2.87	0.092	0.198	0.870

Regression equations	R^2	Vertex *C*
Feed intake in the starter phase = $92.5852 + 17.0598C - 4.0740C^2$	0.52	2.09
FCR in the starter phase = $2.3567 + 0.4405C - 0.1009C^2$	0.46	2.18
Feed intake in the grower phase = $223.5173 + 2.7846C$	0.28	-

BW, body weight; FCR, feed conversion ratio; *C*, extract concentration.

V. CONSIDERAÇÕES FINAIS

Os presentes resultados mostraram que a a inclusão do extrato de Tag na dieta de reprodutores, aos níveis indicados pelo fabricante como pigmentante de gemas de ovos, não apresenta características suficientes para melhorias de desempenho produtivo, ou como potencial antioxidante do soro e dos tecidos avaliados ou em otimizar a produtividade em se tratando de incubação e desempenho da progênie, quando os animais foram criados em condições ideais de ambiente e temperatura.

A estimativa de fertilidade dos ovos indicou que a inclusão do extrato de Tag desempenha um papel importante na nutrição dos reprodutores, e que esta pode ser uma ferramentta importante para a melhoria desta variável. A suplementanção de extrato de Tag na dieta de reprodutores de codornas japonesas, nos níveis indicados comercialmente, apresentam uma boa capacidade de pigmentação, deposição de carotenoides e xantofilas nas gemas, o que pode proporcionar como objetivo final pintainhos de melhor qualidade.