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FITASE SUPRANUTRICIONAL COMO FERRAMENTA PARA OTIMIZAÇÃO DO APROVEITAMENTO DO FÓSFORO EM POEDEIRAS LEVES

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Porto Alegre

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FITASE SUPRANUTRICIONAL COMO FERRAMENTA PARA OTIMIZAÇÃO DO APROVEITAMENTO DO FÓSFORO EM POEDEIRAS LEVES

Tese apresentada ao Programa de Pós-Graduação em Zootecnia, Área de Concentração em Nutrição e Metabolismo Animal, como requisito para obtenção do Grau de Doutora em Zootecnia, na Faculdade de Agronomia, da Universidade Federal do Rio Grande do Sul.

Orientador: Sergio Luiz Vieira

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“Nossas dúvidas são traidoras e nos fazem perder o que, com frequência, poderíamos ganhar, por simples medo de arriscar.” (*William Shakespeare*)

RESUMO[1]

O objetivo do estudo foi avaliar os efeitos de níveis crescentes de fósforo disponível (Pd) e da suplementação de fitase sobre o desempenho produtivo, qualidade dos ovos, digestibilidade e retenção de nutrientes, características ósseas e biomarcadores do metabolismo mineral de 324 poedeiras Bovans White das 26 às 57 semanas de idade. As aves foram distribuídas em delineamento inteiramente casualizado em esquema fatorial 2 × 6 (Fitase: 0 e 4.000FYT/kg x Pd: 0,09, 0,16, 0,23, 0,30, 0,37 e 0,44%), totalizando 12 tratamentos com 27 repetições cada. O desempenho e a qualidade dos ovos foram avaliados em oito períodos de 28 dias, enquanto a digestibilidade ileal, retenção de nutrientes, características da tíbia, parâmetros séricos, hormônios plasmáticos e concentração de fosvitina na gema foram determinados ao final do experimento. Os dados foram analisados utilizando os PROC's MIXED e GLM do SAS, considerando efeitos lineares e quadráticos dos níveis de Pd. Foram observadas interações entre fitase e Pd para ganho de peso (GP), produção de ovos, conversão alimentar (CA), digestibilidade de matéria seca, proteína bruta e energia, características ósseas, retenção de fósforo (P), energia metabolizável aparente (AME), atividade sérica da fosfatase alcalina (FA) e concentração de fosvitina na gema, demonstrando que a resposta das aves ao P dietético foi modificada pela suplementação enzimática. Independentemente dos níveis de Pd, a fitase aumentou o GP (+16,3%), a produção de ovos (+2,0%), o número total de ovos (+4 ovos/ave), o peso dos ovos (+0,5 g), a digestibilidade ileal do P (+23,6%), a retenção de P (+27,3%), a AME (+234 kcal/kg), a resistência da tíbia (+16,9%) e a concentração de fosvitina na gema (+96,2%). Além disso, elevou as concentrações séricas de cálcio (+26,6%) e P (+27,0%), bem como o paratormônio plasmático (+60,1%). Conclui-se que a suplementação de 4.000 FYT/kg de fitase é uma estratégia eficaz para reduzir a exigência de Pd de aproximadamente 0,37 para 0,23% em poedeiras comerciais, sem comprometer o desempenho produtivo.

Palavras-chave: Poedeiras. Fósforo. Fitase. Desempenho. Metabolismo mineral

[1]Tese de Doutorado em Zootecnia - Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (127 p.) Julho, 2026.

ABSTRACT[1]

The objective of this study was to evaluate the effects of increasing dietary available phosphorus (avP) levels and phytase supplementation on productive performance, egg quality, nutrient digestibility and retention, bone characteristics, and biomarkers of mineral metabolism in 324 Bovans White laying hens from 26 to 57 weeks of age. Birds were assigned to a completely randomized design in a 2 × 6 factorial arrangement (phytase: 0 and 4,000 FYT/kg × avP: 0.09, 0.16, 0.23, 0.30, 0.37, and 0.44%), totaling 12 treatments with 27 replicates each. Productive performance and egg quality were evaluated over eight consecutive 28-day periods, whereas ileal digestibility, nutrient retention, tibia characteristics, serum parameters, plasma hormones, and yolk phosvitin concentration were determined at the end of the experiment. Data were analyzed using the MIXED and GLM procedures of SAS, considering linear and quadratic effects of avP levels. Significant phytase × avP interactions were observed for body weight gain (BWG), egg production, feed conversion ratio (FCR), dry matter, crude protein and energy digestibility, bone characteristics, phosphorus (P) retention, apparent metabolizable energy (AME), serum alkaline phosphatase activity, and yolk phosvitin concentration, demonstrating that the birds' response to dietary P was modified by enzyme supplementation. Regardless of avP level, phytase increased BWG (+16.3%), egg production (+2.0%), total egg production (+4 eggs/hen), egg weight (+0.5 g), ileal P digestibility (+23.6%), P retention (+27.3%), AME (+234 kcal/kg), tibia breaking strength (+16.9%), and yolk phosvitin concentration (+96.2%). In addition, phytase increased serum calcium (+26.6%) and P (+27.0%) concentrations, as well as plasma parathyroid hormone (+60.1%). It was concluded that supplementation with 4,000 FYT/kg phytase is an effective strategy to reduce the avP requirement from approximately 0.37 to 0.23% in commercial laying hens without compromising productive performance.

Keywords: Layers. Phosphorus. Phytase. Performance. Mineral metabolism.

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1. INTRODUÇÃO

O fósforo (P) é o segundo mineral mais abundante no organismo animal e desempenha funções essenciais relacionadas à mineralização óssea, metabolismo energético, integridade celular e manutenção da homeostase ácido-base e osmótica (SUTTLE, 2022; QADEER; BASHIR, 2026). Em aves de postura, sua importância é ainda maior devido à elevada demanda metabólica associada à produção contínua de ovos, que requer o constante fornecimento de minerais para a formação da casca e manutenção da integridade esquelética (NYS; LE ROY, 2018; SINCLAIR-BLACK et al., 2023). Aproximadamente 80 a 85% do P corporal encontra-se depositado nos ossos na forma de hidroxiapatita, constituindo uma importante reserva mineral que pode ser mobilizada para atender às exigências fisiológicas durante os ciclos produtivos (BONJOUR, 2011; QADEER; BASHIR, 2026).

Apesar de ingredientes amplamente utilizados na alimentação de aves, como milho e farelo de soja, apresentarem quantidades consideráveis de P, grande parte desse mineral encontra-se complexada ao ácido fítico, tornando-se pouco disponível devido à limitada atividade de fitase endógena no trato gastrointestinal (RAVINDRAN et al., 1995; SELLE; RAVINDRAN, 2007). Como consequência, aumenta-se a dependência de fontes inorgânicas de P e a excreção desse mineral no ambiente (CORDELL; DRANGERT; WHITE, 2009; WALDROUP, 1999). Nesse contexto, a suplementação de fitase microbiana tornou-se uma estratégia nutricional amplamente utilizada para aumentar a hidrólise do fitato, melhorar a disponibilidade de P e reduzir sua excreção. Além disso, a fitase pode aumentar a utilização de outros nutrientes associados à molécula de fitato, como cálcio (Ca), aminoácidos e energia (RAVINDRAN et al., 2000; SEBASTIAN et al., 1996; COWIESON et al., 2004).

As respostas à suplementação de fitase em poedeiras apresentam particularidades em relação às observadas em frangos de corte. As elevadas concentrações de Ca presentes nas dietas de poedeiras favorecem a formação de complexos Ca-fitato pouco solúveis, reduzindo a disponibilidade do substrato para a ação enzimática (TAMIM; ANGEL; CHRISTMAN, 2004). Além disso, o metabolismo mineral dessas aves é caracterizado por intensa remodelação óssea e por mecanismos fisiológicos especializados que regulam a absorção,

retenção, mobilização e deposição de Ca e P para atender simultaneamente às demandas do esqueleto e da produção de ovos (DACKE et al., 1993; NYS; LE ROY, 2018; SINCLAIR-BLACK et al., 2023).

A determinação precisa das exigências de P em poedeiras modernas tornou-se um tema de crescente interesse devido aos avanços genéticos que aumentaram significativamente a persistência produtiva e a longevidade das aves. Embora diversos estudos tenham proposto níveis de P disponível para poedeiras, os resultados permanecem inconsistentes em função de diferenças entre linhagens, idade das aves, composição das dietas, duração experimental e níveis de suplementação de fitase avaliados. Dessa forma, o estabelecimento de recomendações atualizadas depende da compreensão das respostas produtivas e fisiológicas das aves às diferentes estratégias de suplementação.

Além de sua importância para o desempenho produtivo, o P desempenha papel fundamental na manutenção da integridade esquelética e da homeostase mineral. Alterações no suprimento desse nutriente podem comprometer a mineralização e a resistência óssea, desencadeando adaptações fisiológicas relacionadas ao metabolismo mineral (BAR; HURWITZ, 1984; NIE et al., 2018; WEI et al., 2022). Adicionalmente, o P é depositado no ovo, principalmente na forma de fosvitina, a principal fosfoproteína da gema, responsável pelo armazenamento de P e pela ligação de minerais essenciais ao desenvolvimento embrionário. Assim, a disponibilidade de P pode influenciar tanto a qualidade óssea das aves quanto a deposição de compostos fosforados no ovo (BURLEY; COOK, 1961; SAMARAWEERA et al., 2011).

Diante desse contexto, o presente estudo teve como objetivo avaliar os efeitos de níveis crescentes de P disponível, associados ou não à suplementação com uma dose supranutricional de fitase (4.000 FYT/kg), sobre o desempenho produtivo, a qualidade dos ovos, a digestibilidade e retenção de nutrientes, a mineralização óssea e os indicadores fisiológicos relacionados ao metabolismo mineral de poedeiras comerciais ao longo do ciclo de produção. Adicionalmente, buscou-se determinar as exigências de fosforo disponível para galinhas poedeiras em um período experimental de 32 semanas pós-pico de produção.

2. REVISÃO BIBLIOGRÁFICA

2.1. FÓSFORO NA NUTRIÇÃO DE POEDEIRAS

O P é um dos minerais mais abundantes e importantes no organismo animal, sendo considerado o segundo mineral mais presente no corpo das aves, atrás apenas do Ca (SUTTLE, 2022). Sua importância vai além da formação óssea, participando de diversos processos fisiológicos essenciais para a manutenção da vida, do crescimento e da reprodução. Em poedeiras comerciais, a adequada nutrição fosfórica assume papel ainda mais relevante devido à elevada demanda metabólica associada à produção contínua de ovos. Dessa forma, o fornecimento adequado de P é fundamental para garantir desempenho produtivo, integridade esquelética e eficiência na utilização dos nutrientes (KESHAVARZ, 2000; ENGLMAIEROVÁ; SKŘIVANOVÁ; SKŘIVAN, 2014).

A maior parte do P corporal, aproximadamente 80 a 85%, encontra-se depositada nos ossos na forma de hidroxiapatita, juntamente com o Ca, contribuindo para a resistência e sustentação do esqueleto (BONJOUR, 2011; FUKUMOTO, 2014). Além de sua função estrutural, o P participa ativamente do metabolismo energético por meio de compostos fosforilados, como o trifosfato de adenosina (ATP), principal molécula responsável pelo armazenamento e transferência de energia nos sistemas biológicos (SUTTLE, 2010). O P também integra a composição dos ácidos nucleicos (DNA e RNA), dos fosfolipídios presentes nas membranas celulares e de diversas moléculas envolvidas em processos metabólicos e de sinalização intracelular (SUTTLE, 2022). Dessa forma, sua disponibilidade adequada é indispensável para o funcionamento normal dos tecidos e órgãos.

Nas poedeiras, a importância do P é ampliada pela necessidade contínua de produção e deposição de minerais tanto nos ossos quanto nos ovos. Durante a postura, ocorre intensa mobilização mineral para sustentar a formação da casca, processo que exige mecanismos fisiológicos eficientes de absorção, armazenamento e reutilização de minerais (DACKE et al., 1993; FLEMING et al., 1994). Embora a casca seja composta predominantemente por Ca, a manutenção da homeostase mineral depende diretamente do adequado suprimento de P, uma vez que ambos os minerais apresentam estreita interação metabólica (KESHAVARZ, 1986). Dietas deficientes em P podem comprometer

não apenas o metabolismo ósseo, mas também o desempenho produtivo e a qualidade dos ovos (BAR; HURWITZ, 1984; NIE et al., 2018).

O P presente nos alimentos pode ser expresso de diferentes formas. O P total representa a quantidade total do mineral presente no ingrediente ou na dieta, independentemente de sua disponibilidade para o animal. Entretanto, nem todo o P ingerido pode ser efetivamente utilizado pelas aves. Em ingredientes de origem vegetal, como milho e farelo de soja, grande parte do P encontra-se armazenada na forma de fitato, composto cuja utilização é limitada devido à baixa atividade de fitases endógenas no trato gastrointestinal das aves (SHASTAK et al., 2012; RODEHUTSCORD, 2013; WOYENGO; NYACHOTI, 2013). Como consequência, apenas uma fração do P total está disponível para absorção e utilização metabólica.

Diante dessa limitação, passou-se a utilizar o conceito de P disponível, que representa a fração do P capaz de atender às exigências fisiológicas do animal (RODEHUTSCORD, 2009). Mais recentemente, o conceito de P digestível ganhou destaque por permitir estimativas mais precisas da quantidade efetivamente absorvida pelo trato digestório (SHASTAK et al., 2012). Sistemas baseados em P digestível apresentam vantagens por considerar diferenças na digestibilidade entre ingredientes e por permitirem formulações nutricionais mais precisas, contribuindo para a redução da excreção de P e para a melhoria da eficiência alimentar (BIKKER et al., 2016).

Para suprir as exigências nutricionais das aves, é comum a utilização de fontes inorgânicas de P nas formulações, principalmente fosfato bicálcico e fosfato monocálcico. Essas fontes apresentam elevada disponibilidade biológica e são utilizadas para complementar o P fornecido pelos ingredientes vegetais, garantindo adequado desempenho produtivo e mineralização óssea. Entretanto, os fosfatos inorgânicos representam uma das matérias-primas minerais de maior custo na formulação das dietas e sua utilização excessiva pode aumentar a excreção de P no ambiente, estimulando a busca por estratégias que melhorem o aproveitamento do P naturalmente presente nos ingredientes vegetais (LAMP et al., 2020; LEE; LOPEZ; STEIN, 2022; WANG et al., 2022).

A deficiência de P pode resultar em diversas alterações fisiológicas e produtivas. Em poedeiras, níveis insuficientes desse mineral geralmente estão associados à queda na produção e qualidade dos ovos, piora na conversão

alimentar e diminuição da qualidade óssea (BOLING et al., 2000; ZHANG et al., 2020; GRANGHELLI; PERSIA, 2022). Como resposta à menor disponibilidade dietética de P, ocorre aumento da mobilização mineral dos ossos, especialmente do osso medular, com o objetivo de manter as concentrações circulantes necessárias para as funções vitais e para a produção dos ovos (BELLO; DERSJANT; KORVER, 2020; Wei et al., 2022 REN et al., 2023). Por outro lado, o excesso de P também pode ser prejudicial, pois pode interferir no metabolismo do Ca, aumentar os custos de formulação das dietas e elevar a excreção desse mineral no ambiente, contribuindo para problemas ambientais relacionados à eutrofização (CORDELL; DRANGERT; WHITE, 2009; LI; ZHANG; BRYDEN, 2017).

A adequada nutrição fosfórica é essencial para sustentar o desempenho produtivo das poedeiras modernas. Diversos estudos demonstram que níveis adequados de P favorecem o consumo de ração, a produção de ovos, o peso dos ovos e a eficiência alimentar, além de contribuírem para a manutenção da integridade óssea ao longo do ciclo produtivo (RODEHUTSCORD et al., 2022; PAGNUSSATT et al., 2025). Considerando sua ampla participação nos processos metabólicos, produtivos e estruturais das aves, o P permanece como um dos nutrientes de maior importância na alimentação de poedeiras, tornando sua utilização eficiente um dos principais desafios da nutrição avícola moderna.

2.2. METABOLISMO MINERAL

A absorção intestinal de P em aves ocorre predominantemente ao longo do intestino delgado por meio de duas vias principais: a transcelular (ativa), mediada por co-transportadores específicos como NaPi-IIb, PiT-1 e PiT-2, e a paracelular (passiva), que ocorre por difusão simples. Esse processo dinâmico é modulado por fatores ontogenéticos (idade), variações na composição dietética e pelas demandas homeostáticas do estado fisiológico animal (HUBER; ZELLER RODEHUTSCORD, 2015; HU et al., 2018). Após sua absorção, o mineral circula principalmente na forma de fosfato inorgânico e é distribuído para os diferentes tecidos de acordo com as demandas metabólicas (QADEER; BASHIR, 2023). Além de sua participação na estrutura óssea, o P é componente fundamental de moléculas envolvidas no armazenamento e transferência de energia, como o ATP, bem como dos ácidos nucleicos e fosfolipídios de membrana, participando diretamente da manutenção da atividade celular (SUTTLE, 2022).

Nas poedeiras, o metabolismo do P assume importância adicional devido à elevada demanda mineral associada à produção contínua de ovos (WHITEHEAD; FLEMING, 2000). Embora a casca seja composta predominantemente por carbonato de Ca, a manutenção da integridade óssea e a reposição das reservas minerais dependem do adequado suprimento de P (RODEHUTSCORD et al., 2022). Durante a postura ocorre intenso processo de remodelação óssea, no qual o osso medular atua como uma reserva mineral temporária capaz de fornecer rapidamente Ca e P para a circulação. Como esses minerais estão associados nos cristais de hidroxiapatita, a reabsorção óssea resulta na liberação simultânea de ambos para atender às exigências fisiológicas da ave (DACKE, 1993; NYS; LE ROY, 2018).

A manutenção das concentrações circulantes de Ca e P depende da integração entre intestino, rins e tecido ósseo, que atuam continuamente para equilibrar absorção, retenção, mobilização e excreção mineral. Durante a formação da casca, a elevada demanda por Ca reduz sua concentração plasmática, desencadeando mecanismos hormonais que estimulam a mobilização de minerais armazenados no esqueleto e aumentam a absorção intestinal desses nutrientes. Esse processo garante o fornecimento adequado de minerais para a produção dos ovos sem comprometer imediatamente as concentrações circulantes necessárias às demais funções fisiológicas (DACKE, 1993; VEUM, 2010; PROSZKOWIEC-WEGLARZ; ANGEL, 2013; NYS; LE ROY, 2018).

Entre os principais reguladores da homeostase mineral destacam-se o paratormônio (PTH), a vitamina D e, mais recentemente, o fator de crescimento de fibroblastos 23 (FGF23). O PTH é secretado em resposta à redução das concentrações plasmáticas de Ca e estimula a mobilização mineral do tecido ósseo, além de atuar sobre os rins e favorecer a síntese da forma biologicamente ativa da vitamina D (TAYLOR; BELANGER, 1969; SINCLAIR-BLACK; GARCIA; ELLESTAD, 2023). Por sua vez, a vitamina D aumenta a absorção intestinal de Ca e P, contribuindo para a manutenção do equilíbrio mineral e para os processos de mineralização óssea (SPENCER et al., 1978; NYS; LE ROY, 2018). O FGF23, produzido principalmente pelo tecido ósseo, atua como um importante regulador do metabolismo fosfático, participando do controle das concentrações circulantes de P e da sua excreção renal (REN et al., 2020;

SINCLAIR-BLACK; GARCIA; ELLESTAD, 2023). Estudos recentes indicam que esse hormônio desempenha papel central na manutenção da homeostase do P em poedeiras, especialmente durante períodos de intensa remodelação óssea e elevada atividade produtiva (GLOUX et al., 2020; REN et al., 2019a).

Devido à estreita interação metabólica entre Ca e P, o aproveitamento eficiente desses minerais depende não apenas de seus níveis dietéticos individuais, mas também do equilíbrio entre eles. Alterações na relação Ca:P podem comprometer a absorção, retenção e utilização de ambos os nutrientes, afetando a mineralização óssea, a qualidade da casca e o desempenho produtivo das aves (Proszkowiec-Weglarz; Angel, 2013; LI; ZHANG; BRYDEN, 2017). Dessa forma, a compreensão dos mecanismos que regulam a homeostase mineral constitui a base para o desenvolvimento de estratégias nutricionais capazes de sustentar simultaneamente a saúde óssea e a produção de ovos ao longo de todo o ciclo produtivo.

2.3. FITASE E FITATO

Grande parte do P presente nos ingredientes vegetais utilizados na alimentação de aves encontra-se armazenada na forma de fitato, também denominado ácido fítico ou mio-inositol hexafosfato (IP_6) (SELLE; RAVINDRAN, 2007). Em ingredientes como milho e farelo de soja, aproximadamente dois terços do P total estão associados a essa molécula (RAVINDRAN; BRYDEN; KORNEGAY, 1995). Entretanto, devido à reduzida atividade de fitases endógenas no trato gastrointestinal das aves, apenas uma pequena fração desse P pode ser aproveitada, tornando necessária a suplementação das dietas com fontes inorgânicas de P para atender às exigências nutricionais dos animais (NRC, 1994; SELLE; RAVINDRAN, 2007). Como consequência, parte significativa do P consumido é excretada, aumentando os custos de formulação e os impactos ambientais associados à produção animal (WALDROUP, 1999).

Além de limitar a disponibilidade de P, o fitato é reconhecido como um importante fator antinutricional em dietas para monogástricos. Sua elevada densidade de cargas negativas favorece a formação de complexos com minerais, especialmente Ca, zinco, ferro e manganês, além de proteínas e enzimas digestivas, reduzindo a digestibilidade e a absorção de diversos nutrientes. Dessa forma, os efeitos do fitato vão além da restrição do aproveitamento do P, podendo comprometer a utilização de aminoácidos,

energia e minerais (VOHRA; GRAY; KRATZER, 1965; SEBASTIAN et al., 1996; COWIESON; ACAMOVIC; BEDFORD, 2007; RAVINDRAN et al., 2010).

A intensidade desses efeitos é influenciada por fatores como o teor de fitato da dieta, o pH do trato gastrointestinal, a idade das aves e, principalmente, a concentração de Ca e a relação Ca:P da dieta (SELLE; RAVINDRAN, 2007). Em condições de elevada concentração de Ca, ocorre maior formação de complexos Ca-fitato pouco solúveis, reduzindo a degradação do fitato e a disponibilidade do P (LIM; NAMKUNG; PAIK, 2003; TAMIM; ANGEL; CHRISTMAN, 2004). Esse aspecto é particularmente relevante em poedeiras, cujas dietas apresentam concentrações elevadas de Ca para atender à demanda mineral associada à produção contínua de ovos (HENDRIX, 2020).

A suplementação de fitase exógena constitui a principal estratégia para aumentar a utilização do P fitático. Essa enzima promove a hidrólise sequencial dos grupos fosfato ligados ao anel de mio-inositol, liberando P inorgânico para absorção intestinal e reduzindo a formação de complexos fitato-nutriente (ZELLER et al., 2015; SOMMERFELD et al., 2018). Como resultado, além de aumentar a disponibilidade de P, a fitase pode melhorar a digestibilidade de aminoácidos, energia e minerais, favorecendo a eficiência de utilização da dieta e reduzindo a necessidade de suplementação com fosfatos inorgânicos (YI et al., 1996; RAVINDRAN et al., 2000; COWIESON; ACAMOVIC; BEDFORD, 2004).

Em poedeiras, entretanto, as respostas à suplementação com fitase podem variar em função da idade das aves, da composição da dieta, da fonte e granulometria do calcário, do nível de P dietético e da dose da enzima utilizada (DERSJANT-LI et al., 2015). Tradicionalmente, a fitase foi utilizada em níveis nutricionais, geralmente entre 300 e 1.000 FTU/kg de ração, visando principalmente à liberação de parte do P fitático e à redução da inclusão de fontes inorgânicas de P (COWIESON; WILCOCK; BEDFORD, 2011; DERSJANT-LI et al., 2015). Contudo, avanços no desenvolvimento enzimático e uma melhor compreensão dos efeitos antinutricionais do fitato estimularam a utilização de doses superiores às convencionalmente recomendadas (COWIESON; WILCOCK; BEDFORD, 2011)

Nessa abordagem, busca-se promover uma degradação mais extensa do fitato, reduzindo a concentração residual de IP_6 no trato gastrointestinal e

potencializando não apenas a liberação de P, mas também a redução dos efeitos antinutricionais da molécula (ZELLER et al., 2015). Como consequência, podem ser observadas melhorias adicionais na digestibilidade de nutrientes, na utilização de minerais e no aproveitamento energético da dieta (SELLE; RAVINDRAN, 2007; SOMMERFELD et al., 2018). Em poedeiras, o uso de fitase em doses supranutricionais tem despertado crescente interesse devido ao potencial de aumentar a eficiência de utilização do P em dietas com elevada inclusão de Ca, reduzindo a dependência de fosfatos inorgânicos e contribuindo para sistemas de produção mais sustentáveis (BOLING et al., 2000)

2.4. BIOMARCADORES DO METABOLISMO FOSFÓRICO

A avaliação do metabolismo fosfórico em poedeiras pode ser realizada por meio de biomarcadores sanguíneos e ósseos que refletem a disponibilidade de minerais, a atividade metabólica do tecido ósseo e os mecanismos de regulação da homeostase mineral. Entre os principais indicadores utilizados destacam-se as concentrações séricas de Ca e P, a atividade da fosfatase alcalina (FA), os níveis de PTH e CT, a concentração de fosvitina na gema e a composição mineral do tecido ósseo. As concentrações séricas de Ca e P representam indicadores da disponibilidade imediata desses minerais na circulação. Entretanto, devido aos mecanismos homeostáticos que regulam rigorosamente suas concentrações plasmáticas, alterações nesses parâmetros normalmente refletem mudanças mais expressivas no metabolismo mineral ou no fornecimento dietético de nutrientes (NYS; LE ROY, 2018).

Em poedeiras, a interpretação desses indicadores deve considerar as variações fisiológicas associadas ao ciclo ovulatório, uma vez que as demandas minerais para a formação da casca promovem oscilações nos fluxos de absorção intestinal, deposição óssea e mobilização mineral ao longo do dia (REN et al., 2019B). A FA é uma enzima produzida principalmente pelos osteoblastos e está diretamente relacionada aos processos de formação e mineralização óssea. Sua atividade é frequentemente utilizada como indicador da intensidade do metabolismo ósseo, sendo geralmente associada a maiores taxas de deposição mineral e remodelação do tecido esquelético (GOLUB; BOESZE-BATTAGLIA, 2007; MILLÁN, 2013).

O PTH e a CT atuam de forma complementar na regulação da homeostase mineral. O PTH é secretado em resposta à redução das

concentrações circulantes de Ca (VAN DE VELDE; LOVERIDGE; VERMEIDEN, 1984a; SINGH et al., 1986) e estimula mecanismos que aumentam a disponibilidade de minerais para o organismo, incluindo a mobilização óssea (TAYLOR; BELANGER, 1969) e a ativação da vitamina D (BRENZA; DELUCA, 2000).

Em aves, a CT não afeta a atividade dos osteoclastos em condições normais de saúde. Contudo, sob condições de deficiência crônica de cálcio e vitamina D3, observa-se a expressão induzida de receptores de calcitonina nos osteoclastos, permitindo que o hormônio atue reduzindo a reabsorção óssea apenas nesse cenário de estresse mineral (DE VERNEJOUL et al., 1988). Em poedeiras, a atuação desses hormônios é fundamental para atender às elevadas demandas minerais associadas à formação da casca dos ovos. A composição mineral dos ossos, especialmente as concentrações de Ca e P, constitui um importante indicador do estado nutricional das aves em relação ao metabolismo mineral (BOBECK; CHO; COOK, 2012; REN; PAYNE; COOK, 2017). Como o tecido ósseo representa a principal reserva corporal desses minerais, alterações em sua composição refletem o balanço entre deposição e mobilização mineral ao longo do ciclo produtivo (VAN DE VELDE et al., 1984b; WILSON; DUFF, 1990; KERSCHNITZKI et al., 2014).

Outro biomarcador que tem recebido atenção crescente é a fosvitina, uma proteína da gema caracterizada por seu elevado teor de grupos fosfato e considerada a principal fosfoproteína do ovo (MECHAM; OLCOTT, 1949; YAMAMOTO et al., 1997). Além de atuar como reserva de P para o desenvolvimento embrionário, sua concentração pode refletir alterações no metabolismo e na disponibilidade desse mineral para a ave (LI et al., 2014; SAMARAWEERA et al., 2011). Dessa forma, a avaliação da fosvitina pode fornecer informações complementares sobre a utilização e o direcionamento do P absorvido para a formação do ovo.

Em conjunto, esses biomarcadores permitem avaliar diferentes aspectos do metabolismo fosfórico. Enquanto Ca e P séricos refletem a disponibilidade mineral circulante, FA, PTH e CT estão relacionados aos mecanismos de regulação e remodelação óssea. Já a composição mineral dos ossos e a concentração de fosvitina fornecem informações sobre o destino e o

armazenamento de P no organismo, complementando a avaliação das respostas fisiológicas à nutrição fosfórica.

3. HIPÓTESES E OBJETIVOS

3.1. HIPÓTESES

A suplementação de fitase em dose supranutricional aumenta a disponibilidade e a utilização do fósforo, melhorando o desempenho produtivo e os parâmetros relacionados ao metabolismo mineral.

O aumento das concentrações de fósforo disponível na dieta promove melhorias no desempenho produtivo, na qualidade dos ovos e na mineralização óssea das poedeiras.

A suplementação de fitase em dietas com baixos níveis de fósforo disponível é capaz de compensar parcialmente a deficiência desse mineral, mantendo as respostas produtivas e fisiológicas das aves.

3.2. OBJETIVO GERAL

Avaliar os efeitos da suplementação de fitase em dose supranutricional (4.000 FYT/kg) e de diferentes concentrações de fósforo disponível na dieta sobre o desempenho produtivo, qualidade dos ovos, digestibilidade e utilização dos nutrientes, mineralização óssea e metabolismo mineral em poedeiras comerciais leves (Bovans White).

3.3. OBJETIVO ESPECÍFICO

Avaliar os efeitos da fitase e dos níveis de fósforo disponível sobre o desempenho produtivo e qualidade de ovos de galinhas poedeiras.

Avaliar a digestibilidade ileal e retenção do fósforo, cálcio e energia.

Determinar os efeitos dos tratamentos sobre a mineralização óssea.

Avaliar biomarcadores sanguíneos e hormonais relacionados ao metabolismo do fósforo e do cálcio.

Avaliar a concentração de fosvitina na gema como indicador da deposição e utilização do fósforo no ovo.

Determinar a exigência de fósforo para galinhas poedeiras após o pico de produção.

4. ARTIGO I

SUBMETIDO NA REVISTA POULTRY SCIENCE EM JUNHO DE 2026

Supranutritional phytase and dietary phosphorus effects on White Leghorns: I. Performance and egg traits

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ABSTRACT

This study evaluated the effects of supranutritional phytase supplementation (4,000 FYT/kg) in feeds having varying dietary phosphorus (P) on performance, egg traits, and ileal digestibility of White Leghorns (Bovans White) hens from 26 to 57 wk of age. A total of 324 hens were assigned to a 2 x 6 factorial: feeds with or without phytase vs. six graded increases of dietary P (avP = 0.09 to 0.44% equivalent to 0.07 to 0.31% digestible P). Responses were evaluated over eight 28 d periods; ileal digestibility was determined at the end of the trial. Significant phytase × avP interactions were observed for BW gain (BWG), feed conversion ratio (FCR; kg/kg), egg production, and the digestibility of DM, energy, and CP ($P < 0.05$). Without phytase, BWG increased linearly with increasing avP, whereas FCR (kg/kg) and egg production showed quadratic responses, with optimum values at 0.33 and 0.37% avP, respectively. In phytase-supplemented hens, BWG, FCR (kg/kg), egg production, and energy digestibility responded linearly, whereas DM and CP digestibility showed quadratic responses. Phytase supplementation increased BWG (+16.3%), feed intake (FI; +1.7%), egg production (+2.0%), total eggs (+4 eggs/hen), egg weight (+0.5 g), eggshell proportion (+0.19%), shell breaking strength (+0.17 kgf), shell thickness (+6 μm), and ileal P digestibility (+23.6%), while improving FCR (kg/kg) and reducing yolk percentage ($P < 0.05$). Independently of phytase, FI, eggshell proportion, and shell thickness increased linearly with increasing avP, whereas egg weight was maximized at 0.30% avP. Feed conversion ratio (kg feed/dozen eggs) responded quadratically to avP, with an estimated optimum at 0.34% avP. Calcium digestibility decreased linearly as avP increased, whereas P digestibility was unaffected by avP ($P > 0.05$). In conclusion, supranutritional phytase supplementation modified the response of laying hens to dietary P, reducing the avP concentration required to sustain productive performance from 0.33–0.37% to 0.23–0.27%.

Key words: laying hen, phytase, phosphorus, egg traits, nutrient digestibility.

INTRODUCTION

Phosphorus (P), the second most abundant mineral in the animal body, is indispensable for skeletal development and maintenance. It also plays essential roles in ATP-dependent energy metabolism and the maintenance of acid–base and osmotic homeostasis (Suttle, 2022). Phosphorus deficiency can impair feed intake (FI), thereby compromising egg production (Gordon and Roland, 1998; Nie et al., 2018; Suttle, 2022). Conversely, excessive dietary P may disrupt calcium (Ca) metabolism, reducing its deposition in the eggshell and ultimately compromising shell quality (Roland and Farmer, 1986). Consequently, optimizing dietary P supply is critical for maximizing productivity while maintaining eggshell quality.

Corn and soybean meal (SBM), as well as other plant feedstuffs, contain most of their P in the form of phytate, which is largely unavailable to poultry in the absence of exogenous phytase (Ravindran et al., 1995). This leads to a high proportion of dietary P being excreted rather than retained, increasing feed costs and contributing to P accumulation in agricultural soils following land application of poultry manure, thereby reducing the economic and environmental sustainability of poultry production (Knowlton et al., 2004; Rutherfurd et al., 2004; Jing et al., 2021). Exogenous phytase has become an effective nutritional strategy to hydrolyze phytate, increasing P availability and reducing dependence on inorganic phosphate supplementation.

Despite extensive research demonstrating the efficacy of phytase in broilers, comparatively fewer studies have evaluated its responses in laying hens. Unlike broilers, laying hens consume feeds containing substantially higher Ca concentrations, at wider Ca:P ratios, to sustain continuous eggshell mineralization. These dietary conditions promote the rapid formation of insoluble Ca–phytate complexes, reducing phytate solubility and limiting phytase access to its substrate (Selle and Ravindran, 2007). The unique mineral metabolism

of laying hens, characterized by continuous medullary bone turnover and tightly regulated Ca and P homeostasis, may alter phytate utilization and responses to phytase compared with broilers (Bar, 2009).

The prolonged productive lifespan of modern laying hens has further increased concerns regarding the adequacy of current dietary P recommendations. Daily mobilization of medullary bone for eggshell formation releases both Ca and P into circulation, providing an endogenous source of P that may influence dietary requirements (Bar, 2009). Previous studies have estimated non-phytate P requirements between 0.22 and 0.35% (Gordon and Roland, 1998; Keshavarz, 2000; Granghelli and Persia, 2022; Rodehutscord et al., 2023). However, phytase responses have varied considerably among studies because of differences in hen age, dietary composition, phytase inclusion level, and experimental duration. Furthermore, most available recommendations are based on relatively short-term studies and may not adequately reflect the genetic progress achieved in egg production persistency and egg mass.

Commercial phytase has traditionally been included at approximately 500 FYT/kg in poultry feeds to improve phytate P utilization and reduce the need for inorganic phosphate supplementation (Cowieson et al., 2011; Dersjant-Li et al., 2015). However, under the high-Ca conditions characteristic of commercial layer feeds, conventional phytase doses may not fully overcome the limitations imposed by rapid Ca–phytate complex formation (Ma et al., 2019). Increasing phytase inclusion to supranutritional levels may accelerate phytate hydrolysis before extensive complex formation occurs, thereby maximizing phytate degradation and enhancing the release of P as well as other nutrients associated with the phytate molecule (Cowieson et al., 2011). Indeed, recent studies have evaluated phytase supplementation at both conventional (Musilova et al., 2017; Jlali et al., 2023; Moura et al.,

2023) and supranutritional inclusion levels (Ren et al., 2023; Pirzado et al., 2024). In addition to improving P utilization, high phytase supplementation may increase the availability of trace minerals, amino acids, and energy, thereby improving productive performance and feed efficiency, as previously demonstrated in broilers (Feijó et al., 2023; Maria et al., 2024).

Therefore, the objective of the present study was to evaluate the effects of a wide range of dietary available P concentrations, from the lowest level achievable without inorganic phosphate supplementation to current commercial levels, with or without supplementation of a supranutritional phytase dose (4,000 FYT/kg). Responses were evaluated in terms of productive performance, egg quality traits, and the ileal digestibility of P, Ca, DM, crude protein (CP), and gross energy (GE) in Bovans White laying hens from 26 to 57 wk of age. It was hypothesized that supplementation with 4,000 FYT/kg phytase would maximize phytate degradation under the high dietary Ca concentrations and wide Ca:P ratios characteristic of commercial layer feeds, thereby maintaining productive performance and eggshell quality while improving ileal P digestibility.

MATERIALS AND METHODS

All procedures utilized in the present study were approved by the Ethics and Research Committee of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil (approval number 46471).

Birds Husbandry and Dietary Treatments

A total of 324 Bovans White pullets originating from a commercial pullet farm (Mercoaves Comércio de Aves Ltda, Bom Princípio, RS, Brazil) were individually placed in cages (0.33 m length × 0.46 m width × 0.40 m height) at 13 wks of age. Photoperiodic stimulation started at arrival with a 13L:11D cycle, followed by weekly increases of 30 min until 16L:8D was reached at 19 wks of age and maintained until the end of the trial. Ambient

121 temperature targeted bird comfort and followed Bovan breeders' recommendations (Hendrix,
122 2020).

123 At 26 wks of age, hens were randomly assigned into a 2 × 6 factorial arrangement of
124 feeds not supplemented or with 4,000 FYT/kg of phytase and six dietary P levels (avP=0.09,
125 0.16, 0.23, 0.30, 0.37, and 0.44%, which corresponded to 0.07, 0.12, 0.17, 0.22, 0.26 and
126 0.31% digestible P (dP)) in a total of 12 treatments. For feed formulation purposes, dP values
127 were assigned according to Rodehutscord (2013). Performance and egg production were
128 evaluated in eight 28 d periods (26–29, 30–33, 34–37, 38–41, 42–45, 46–49, 50–53, and 54–
129 57 wk). Each dietary treatment was replicated 27 times, with one individually caged hen as
130 the replicated experimental unit. The lowest available phosphorus (avP) treatment (0.09%
131 avP; 0.07% dP) was formulated without supplemental inorganic phosphate. In order to
132 formulate the additional treatments with higher P, dicalcium phosphate (24% Ca and 19% P)
133 was progressively added to achieve levels approaching those commonly used in commercial
134 layer feeds (0.44% avP; 0.31% dP). Each dietary treatment was manufactured once per
135 experimental period in approximately 140 kg batches, with samples collected and stored at
136 −20 °C for further analyses.

137 Phytase was supplied as a commercial product (HiPhorius™, 40,000 FYT/g;
138 Novonesis A/S, Bagsvaerd, Denmark) and incorporated into the feeds at 100 g/ton,
139 corresponding to 4,000 FYT/kg (4,186 ± 432 FYT/kg analyzed). This is a fourth-generation
140 6-phytase from Citrobacter braakii expressed in Aspergillus oryzae characterized by high
141 thermostability and optimized activity across the pH conditions of the gastrointestinal tract.
142 The phytase inclusion level exceeded the commercial recommendation in order to promote
143 extensive and rapid degradation of phytate in corn and SBM, thereby maximizing P release.
144 The additive was added without assigning nutritional values for P or Ca.

Feed formulations followed commercial standards with sequential corn–soybean meal feeds: Grower II (13–16 wks), Pre-Lay (17–20 wks), Layer I (21–37 wks), and Layer II (38–57 wks). The Grower II feed contained 2,750 kcal/kg AMEn, 17.0% CP, 1.5% Ca, and 0.45% avP (0.32% dP), whereas the Pre-Lay feed provided 2,800 kcal/kg AMEn, 17.3% CP, 2.2% Ca, and 0.50% avP (0.35% dP). The Layer I feed contained 2,860 kcal/kg AMEn, 17.4% CP and 4.1% Ca (3.77% ± 0.33), while the Layer II feed provided 2,860 kcal/kg AMEn, 17.0% CP and 4.2% Ca (3.84 ± 0.13).

All nutrients met Bovans White recommendations (Hendrix, 2020), except for P. The analyzed total P in the feeds were 0.34% ± 0.02, 0.41% ± 0.03, 0.49% ± 0.02, 0.55% ± 0.03, 0.61% ± 0.06, and 0.69% ± 0.04, respectively. An indigestible marker (Celite Corp., Lompoc, CA) was added at 1% in the feeds of the last experimental period (54–57 wks). The limestone used in the experimental feeds had a geometric mean particle size of 633 ± 17 μm, and its solubility was determined according to the method of Kim et al. (2019) at 43.35, 52.16, and 60.55% after 5, 15, and 30 min of incubation, respectively.

Hen Performance Measurements

Productive performance was evaluated at the end of each 28 d period (29, 33, 37, 41, 45, 49, 53, and 57 wks) and included body weight gain (BWG), daily FI, feed conversion ratio (FCR) per dozen eggs and per kg of eggs, egg weight, egg mass, egg production, and total eggs per hen. Eggs were collected four times a day (08:30 am, 11:00 am, 2:00 pm, and 4:00 pm). Ileal digesta was sampled from 15 hens per treatment after euthanasia by cervical dislocation on the last day of the trial. Digesta were gently flushed with distilled water from the intestinal segment extended from Meckel's diverticulum to approximately 2 cm before the ileocecal junction. Samples were placed into plastic containers, immediately frozen in liquid nitrogen, and subsequently stored at −20 °C until lyophilization (Christ Alpha 2-4 LD

169 freeze dryer; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).
170 Both feeds and lyophilized digesta were then ground to pass through a 0.5-mm screen (TE-
171 631/2 knife mill; Tecnal, Piracicaba, SP, Brazil)

172 ***Analysis and calculations***

173 The following analyses were done with feeds and ileal digesta: DM after oven drying
174 at 105 °C (method 934.01; AOAC International, 2006); Ca and P (AOAC, 2019), and acid-
175 insoluble ash (Vogtmann et al., 1975; Choct and Annison, 1992). Crude protein (N × 6.25)
176 was measured by the combustion method (968.06; AOAC International, 2006), and GE was
177 determined using a bomb calorimeter calibrated with benzoic acid as a standard (IKA C2000
178 calorimeter; IKA-Werke GmbH & Co. KG, Staufen, Germany). Phytase activity in the feeds
179 was expressed in phytase yield units (FYT), defined as the activity that releases 1 μmol of
180 inorganic phosphate from 5.0 mM sodium phytate per minute at pH 5.5 and 37 °C (Engelen
181 et al., 1994). Apparent ileal digestibility of Ca, P, GE, and CP, as well as IDE, were calculated
182 using the equations described by Kong and Adeola (2014).

183 ***Egg Trait Measurements***

184 Twenty eggs per treatment were randomly collected during the last two days of each
185 experimental period for egg quality evaluation. Eggshell, albumen, and yolk weights were
186 recorded and expressed as a percentage of whole egg weight. Eggshells were washed with
187 filtered water and dried at 105°C overnight before determining eggshell proportion. Eggshell
188 thickness was measured three times at the equatorial region of each egg using a digital
189 micrometer (Model IP65; Mitutoyo Corp., Kawasaki, Japan), and the average value was used
190 for statistical analysis. Additionally, 20 eggs per treatment were used to determine eggshell
191 breaking strength using a texture analyzer (Model TA.XT.plus Texture Analyzer; Texture

192 Technologies Corp., Hamilton, MA) with a 75-mm (P/75) breaking probe as described by
193 Molino et al. (2015).

194 ***Statistical Analysis***

195 Data were tested for normality and homogeneity of variances using the Shapiro–Wilk
196 and Levene tests, respectively (Levene, 1960; Shapiro and Wilk, 1965). When required, data
197 were transformed using the arcsine square root transformation ($z = \text{asin}(\text{sqrt}(y + 0.5))$)
198 (Ahrens et al., 1990). Analyses were performed using SAS 9.4 (2013), and significance was
199 declared at $P \leq 0.05$.

200 Performance variables measured over time (BW, FI, FCR [kg/kg and kg/dozen], egg
201 production [%], and egg quality traits) were analyzed using PROC MIXED with repeated
202 measures, including phytase as a fixed qualitative factor, avP as a quantitative factor, period
203 as a source of variation, and cage as the experimental unit and random effect. The covariance
204 structure (AR(1)) was selected based on the Akaike information criterion (Littell et al., 1998).
205 Variables not measured over time, including BWG, total egg production, and digestibility
206 variables, were analyzed using PROC GLM. Linear and quadratic effects of avP were
207 evaluated by regression analysis, with avP used as the primary response criterion and
208 corresponding dP values presented for practical feed formulation purposes. Phytase avP
209 equivalency was estimated by linear interpolation of the observed responses between
210 adjacent dietary avP levels in hens not supplemented with phytase, using egg production and
211 FCR as response criteria. When significant quadratic effects were detected, the avP
212 concentration maximizing the response was estimated from the vertex of the equation ($x = -b/2a$).
213 Mean separation was performed using Tukey's test (Tukey, 1991).

214 **RESULTS**

Analyzed total P concentrations closely reflected formulated values and averaged 0.34 ± 0.02, 0.41 ± 0.03, 0.49 ± 0.02, 0.55 ± 0.03, 0.61 ± 0.06, and 0.69 ± 0.04% for feeds formulated to 0.29, 0.35, 0.42, 0.49, 0.55, and 0.62% total P, respectively. These values correspond to 0.09, 0.16, 0.23, 0.30, 0.37, and 0.44% avP, and were respectively equivalent to 0.07, 0.12, 0.17, 0.22, 0.26, and 0.31% dP.

Performance results are presented in Table 2. Significant phytase × avP interactions were observed for BWG ($P = 0.0183$), FCR (kg feed/kg egg mass; $P = 0.0122$), and percent hen-day egg production ($P = 0.0172$). Body weight gain increased linearly with avP, with a greater slope in the absence of phytase ($Y = -10.59 + 601.56x$; $P < 0.0001$) than with its supplementation ($Y = 66.91 + 235.35x$; $P = 0.0215$), corresponding to increases of 42 and 16 g, respectively, for each 0.07 percentage-point increase in avP. Overall, phytase increased final BWG by 16.3% (150 vs. 129 g; $P = 0.0069$). Feed conversion ratio (kg feed/kg egg mass) responded quadratically to avP in the absence of phytase ($Y = 2.0807 - 1.8098x + 2.749x^2$; $P < 0.0001$), with an estimated optimum at 0.33% avP whereas in phytase-supplemented feeds, FCR was affected linearly ($Y = 1.8073 - 0.1685x$; $P = 0.0052$). Overall, phytase improved FCR by 1.7% (1.802 vs. 1.834; $P = 0.0037$). Percent hen-day egg production, in the absence of phytase, responded quadratically to avP ($Y = 78.92 + 70.55x - 96.33x^2$; $P = 0.0004$), with a maximum response at 0.37% avP whereas in phytase-supplemented feeds, egg production increased linearly as avP was higher ($Y = 91.1930 + 6.7908x$; $P < 0.0001$). Overall, phytase increased hen-day egg production by 2 percentage points (96.2 vs. 94.2%; $P < 0.0001$).

The total eggs/hen produced during the experimental period increased linearly both when hens were not supplemented with phytase ($Y = 201.48 + 40.99x$; $P < 0.0001$) and in phytase-supplemented hens ($Y = 212.01 + 16.37x$; $P < 0.0001$). Overall, hens supplemented

with phytase produced approximately four more eggs per hen than those not supplemented (217 vs. 213 eggs/hen; P = 0.0272). Average daily FI (g/hen/day) was increased by phytase supplementation (113.5 with phytase vs. 111.6 without phytase; P = 0.0044) increasing linearly with dietary avP (P = 0.0014) (Y = 109.86 + 7.3825x; P = 0.0088), corresponding to an estimated increase of 0.52 g/hen per day for each 0.07 percentage-point increase in avP. Feed conversion ratio expressed as kg of feed per dozen eggs was not affected by phytase (P = 0.1454) but responded quadratically to avP (P = 0.0102) ($Y = 1.4737 - 0.8238x + 1.2068x^2$; P = 0.0070), with an estimated optimum avP concentration of 0.34% avP.

Egg trait results are presented in Table 3. No phytase × avP interactions were observed (P > 0.05); however, phytase affected all traits, except for albumen percentage, increasing egg weight (+0.5 g; P = 0.0032), eggshell proportion (+0.19%; P < 0.0001), shell breaking strength (+0.17 kgf; P = 0.0022), and shell thickness (+6 μm; P = 0.0002), while decreasing yolk percentage (−0.3%; P = 0.0007). Egg weight responded quadratically to avP (P = 0.0338) ($Y = 59.38 + 14.14x - 23.42x^2$; P = 0.0348), with an estimated optimum at 0.30% avP. Yolk percentage decreased linearly with avP (P = 0.0059) (Y = 28.227 − 1.4933x; P = 0.0061), whereas albumen percentage and shell breaking strength were unaffected (P > 0.05). Eggshell proportion (P = 0.0012) and shell thickness (P = 0.0071) increased linearly with avP (Y = 8.396 + 0.7215x; P = 0.0015; and Y = 358.34 + 30.16x; P = 0.0110, respectively), corresponding to increases of 0.05% and 2.1 μm, respectively, for each 0.07% increase in avP.

Digestibility results obtained at the end of the trial are presented in Table 4. Significant phytase × avP interactions were observed for digestibility of DM (P = 0.0317) and CP (P = 0.0081) as well as for IDEC (P = 0.0152) and IDE (P = 0.0367). For DM digestibility, phytase-supplemented hens showed a quadratic response to avP (Y = 68.76 +

263 57.48x − 273.59x²; P = 0.0238) with an estimated maximum at 0.11% avP, whereas no
264 regression fit was observed in the absence of phytase for avP (P > 0.05). Crude protein
265 digestibility was best fit to Y = 84.72 + 30.25x − 279.42x² (P = 0.0030) in phytase fed hens
266 with an estimated maximum at 0.05% avP, whereas no significant response to avP was
267 observed in the absence of phytase (P > 0.05). In the absence of phytase, neither IDEC nor
268 IDE was affected by dietary P (P > 0.05). In phytase-supplemented feeds, IDEC and IDE
269 increased linearly with increasing P concentration and were fit to Y = 74.29 + 50.17x (P =
270 0.0006) and Y = 2960.35 + 2017.58x (P = 0.0005), respectively.

271 There was no interaction between phytase and avP for the digestibility of P (P >
272 0.05). However, this response was markedly improved by phytase supplementation (72.5 vs.
273 48.9%; P < 0.0001) whereas dietary avP concentration had no significant effects (P > 0.05).
274 As with P, there was no phytase × avP interaction or neither single effect of phytase on Ca
275 digestibility (P = 0.2347); however, this decreased linearly as dietary avP increased (P =
276 0.0430) (Y = 77.16 − 31.24x; P = 0.0007) indicating a reduction of 2.2 % in Ca digestibility
277 for each 0.07% dietary increase in avP.

278 As shown in Table 5, the phytase × AvP interaction demonstrated that phytase
279 partially mitigated the negative effects of severe P deficiency. At the lowest AvP level
280 (0.09%), phytase improved BWG from −167.6 to −48.1 g/hen, increased hen-day egg
281 production from 88.9 to 94.7%, increased total egg production from 201 to 212 eggs/hen,
282 and reduced FCR from 1.946 to 1.834 kg feed/kg egg mass. For nutrient digestibility
283 variables, differences were observed only for the diet containing 0.09% AvP supplemented
284 with phytase, whereas no differences were detected among the remaining dietary P levels.

DISCUSSION

The findings of the present study demonstrated that the responses of laying hens to increasing dietary avP depended on phytase supplementation, as evidenced by the significant phytase × avP interactions observed for BWG, FCR, egg production, and ileal P digestibility. Rather than simply improving performance, phytase modified the magnitude of the response to dietary P, partially compensating for P deficiency at the lowest avP concentrations. This effect was particularly evident in hens fed 0.09% avP, where phytase markedly improved BWG, egg production, and FCR, demonstrating its ability to alleviate the detrimental effects of severe P deficiency. Consequently, the differences between phytase-supplemented and unsupplemented hens progressively diminished as dietary avP increased. This type of response has been previously reported through the release of phytate-bound P and included an increase in FI and egg production (Rutherfurd et al., 2002; Liu et al., 2007), (Teng et al., 2020).

The magnitude of the compensatory effect of supranutritional phytase can be illustrated by its estimated avP equivalency based on productive responses. Using egg production as the response criterion, hens fed without inorganic phosphate supplementation (0.09% avP) but supplemented with phytase achieved a production rate comparable to that of hens fed approximately 0.21% avP without phytase, corresponding to an avP equivalency of about 0.12 percentage units (approximately 0.09% of dP). Likewise, based on FCR (kg feed/kg egg mass), phytase supplementation was equivalent to approximately 0.10 percentage units of avP (approximately 0.07% of dP). These estimates demonstrate that phytase effectively compensated for dietary P deficiency under the most P-limiting conditions, whereas its relative contribution declined as dietary avP increased and P became less limited.

The reductions in FI, BW, and FCR observed under low P conditions are consistent with previous reports demonstrating that dietary P deficiency compromises performance in laying hens (Yan et al., 2001; Teng et al., 2020; Li et al., 2021). Although many of the mechanisms underlying reduced FI remain unclear, evidence from broiler studies suggests that P status may influence appetite through central and peripheral signaling pathways (Aderibigbe et al., 2022). Poorer FCR at lower avP concentrations may also reflect the greater challenge of maintaining Ca and P homeostasis under inadequate mineral supply (Lim et al., 2003). Hens rely heavily on medullary bone resorption to sustain eggshell formation, a process that becomes increasingly important when P availability is limited (Bar and Hurwitz, 1984; Nie et al., 2018; Sinclair-Black et al., 2023). By improving P and Ca availability, phytase may have reduced reliance on bone mineral mobilization, thereby supporting productive performance (Bello et al., 2020; Ren et al., 2023).

Although phytase induced only minor changes in internal egg components, its effects on eggshell quality were well pronounced. The slight reduction in yolk percentage observed with phytase added to the feeds likely reflects a proportional effect associated with increased egg and shell mass rather than a true alteration in yolk deposition, whereas albumen proportion remained unaffected. Hens fed the lowest dietary avP concentrations exhibited poorer eggshell quality, indicating that P supply was insufficient to sustain optimal shell formation. Because the eggshell contains only small amounts of P and is composed predominantly of Ca, this response was likely a consequence of the impaired bone mineral replenishment following the mobilization of Ca for eggshell synthesis. Under inadequate P supply, incomplete restoration of bone mineral reserves may reduce the availability of Ca for subsequent eggshell calcification, thereby compromising shell quality (Dacke et al., 1993; Whitehead, 2004; Li et al., 2018; Yan et al., 2023). The present findings are supported by

previous reports demonstrating impaired eggshell quality in P-deficient feeds for laying hens (Englmaierová et al., 2015; Shao et al., 2025), although responses to dietary P deficiency have varied among studies (Jing et al., 2018; Pongmanee et al., 2020). The positive effects of phytase on shell thickness and breaking strength suggest enhanced release and utilization of Ca and P from phytate complexes, as previously reported by Gordon and Roland (1998). Similar improvements in shell quality and mineral retention have been reported with phytase supplemented from 1,000 to 3,000 FYT/kg, doses higher than usually added, (Eltahan et al., 2023; Lima et al., 2024), reinforcing the role of phytase in improving mineral availability for eggshell formation.

Dietary phytase increased ileal P digestibility by 23.6%, confirming that the enzyme was functionally active under the conditions of the present study. Despite the limited main effects of phytase on some digestibility variables, the significant phytase × avP indicates that phytase efficacy depended on dietary P supply. In contrast, ileal Ca digestibility was influenced only by dietary avP levels, with no detectable response to phytase supplementation or phytase × avP interaction. The relatively wide Ca:P ratio adopted in the present study may have contributed to these responses by promoting the formation of insoluble Ca–phytate and Ca–phosphate complexes, which are known to reduce phytate solubility and depress P digestibility (Tamim et al., 2004; Nahm, 2007; Selle et al., 2009; Liu et al., 2013).

The responses observed for DM, CP, and energy utilization further suggest that phytase improved nutrient availability beyond P release, as has been previously shown (Cowieson et al., 2011; Dersjant-Li et al., 2015). Although Ca digestibility decreased linearly with dietary avP, it was not affected by phytase supplementation, a response also reported by Musilová et al. (2017). In contrast, Oliveira et al. (2018) and Walters et al. (2019) observed

positive phytase effects on Ca digestibility, suggesting that responses depend on dietary characteristics such as Ca and P concentrations, phytate content, Ca ratio, and Ca source.

Collectively, the findings presented in this study indicate that phytase supplementation improved the efficiency of P utilization and improved the response sof laying hens fed proportionally reduced dietary avP. The variability in the dietary P concentrations that optimized all responses further highlights the difficulty of defining a single P requirement and supports the use of phytase as a practical tool to improve nutrient utilization and reduce the need for inorganic phosphate supplementation.

CONCLUSION

The supranutritional phytase supplementation improved productive performance, eggshell quality over a 32 wk of egg production. Significant phytase × avP interactions demonstrated that phytase alleviated the effects of low dietary P supply and modified the response of hens that corresponded to higher dietary P. Feeds without phytase required approximately 0.33 to 0.37% avP to maximize productive responses, whereas supplementation with 4,000 FYT/kg phytase provided an avP equivalency of 0.10 to 0.12 percentage units, indicating that dietary avP concentrations between 0.23 and 0.27% may be sufficient to maintain productive performance while reducing the inclusion of inorganic phosphate in commercial layer feeds added with supranutritional phytase.

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570 **Table 1**. Ingredient and nutrient composition of feeds having supplemental phytase and
571 graded dietary P increases.

Ingredients, % as-is	Layer I (21 to 37)						Layer II (38 to 57)					
	0.09	0.16	0.23	0.30	0.37	0.44	0.09	0.16	0.23	0.30	0.37	0.44
Corn	55.0	54.8	54.6	54.4	54.3	54.1	55.7	55.4	55.3	55.1	54.9	54.7
Soybean meal, 46%	28.7	28.7	28.7	28.8	28.8	28.8	27.6	27.7	27.7	27.7	27.8	27.8
Soybean oil	3.1	3.1	3.2	3.3	3.3	3.4	3.06	3.12	3.19	3.26	3.32	3.39
Dicalcium phosphate	-	0.40	0.80	1.10	1.50	1.8	-	0.34	0.67	1.11	1.44	1.77
Limestone	12.1	11.9	11.6	11.3	11.0	10.8	12.4	12.2	11.9	11.6	11.3	11.1
Salt	0.34						0.37					
Methionine analogue[1]	0.29						0.26					
L-Lysine HCL	0.01						0.01					
L-Threonine	0.03						0.11					
Vitamin and minerals[2]	0.35						0.35					
Choline chloride	0.12						0.13					
Total	100.00						100.00					
AMEn, kcal/kg	2,860						2,860					
Crude protein	17.35						17.00					
Ca	4.1						4.2					
AvP	0.09	0.16	0.23	0.30	0.37	0.44	0.09	0.16	0.23	0.30	0.37	0.44
Dig. P	0.07	0.12	0.17	0.22	0.26	0.31	0.07	0.12	0.17	0.22	0.26	0.31
NPP	0.08	0.14	0.21	0.28	0.34	0.41	0.08	0.14	0.21	0.28	0.34	0.41
Total P[4]	0.29	0.35	0.42	0.49	0.55	0.62	0.29	0.35	0.42	0.49	0.55	0.62
Na	0.17						0.18					
d Lys	0.85						0.83					
d Met	0.50						0.47					
d TSAA	0.76						0.72					
d Trp	0.149						0.19					
d Thr	0.60						0.66					
d Val	0.75						0.73					
Choline, mg/kg	1,700						1,700					
Phytate[5]	0.21						0.20					
Phytase, FYT/kg[6]	4,208±482						4,164±308					

572 [1] Novus (DL-2-hydroxy-(4-methylthio) butanoic acid).
573 [2] Composition per kg of product: vitamin A, 4,250,000 IU; vitamin D3, 1,650,000 IU; vitamin E, 10,000 IU; vitamin K3, 1,500
574 mg; vitamin B1, 1,000 mg; vitamin B2, 2,300 mg; vitamin B6, 1,500 mg; pantothenic acid, 6 g; vitamin B12, 12,500 mcg; nicotinic
575 acid, 20 g; folic acid, 400 mg; biotin, 100 mg; Se, 130 mg; Fe, 22.5 g; Mn, 40 g; Zn, 27.5 g; I, 500 mg; Cu, 3,250 mg; butylated
576 hydroxytoluene, 28.6 g; Rovabio Advance (Adisseo France S.A.S., Antony, France), 142.9 g.
577 [3]Analyzed total P in the feeds with and without phytase: Layer 1, 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03
578 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.
579 [4]Phytate values using NIRS
580 [5]HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.

Table 2. Cumulative performance of laying hens fed supplemental phytase and graded increases in dietary P from 26 to 57 wks of age.

Phytase, FYT/kg[1]	g	kg	g/hen/day	kg/kg	kg/dozen	%	Units
	BWG[3]	BW	FI	FCR	FCR	Egg production (hen/day)	Total eggs/ hen
None	129[b]	1.61[b]	111.6[b]	1.834[a]	1.352	94.2[b]	213[b]
4,000	150[a]	1.65[a]	113.5[a]	1.802[b]	1.341	96.2[a]	217[a]
AvP, %[2]							
0.09	66[d]	1.59[b]	110.0[b]	1.890[a]	1.377[a]	91.8[c]	206[b]
0.16	95[c]	1.65[a]	112.8[a]	1.850[b]	1.367[ab]	94.2[b]	213[ab]
0.23	125[b]	1.62[ab]	122.8[a]	1.796[c]	1.336[b]	95.9[ab]	215[ab]
0.30	154[a]	1.63[ab]	112.4[a]	1.780[c]	1.332[b]	95.9[ab]	216[a]
0.37	183[a]	1.62[ab]	113.5[a]	1.795[c]	1.335[b]	96.7[a]	218[a]
0.44	213[a]	1.67[a]	113.6[a]	1.795[c]	1.334[b]	96.8[a]	218[a]
SEM	0.010	0.003	0.196	0.004	0.002	0.151	0.158
Probabilities							
Phytase	0.0069	0.0147	0.0044	0.0037	0.1454	<.0001	0.0272
AvP linear	<.0001	0.0088	0.0014	<.0001	0.0001	<.0001	<.0001
AvP quadractic	0.4423	0.9089	0.2141	<.0001	0.0102	<.0001	0.0007
Phytase*AvP linear	0.0183	0.1978	0.0571	0.0466	0.1489	0.0002	0.0002
Phytase*AvP quadratic	0.4208	0.9178	0.4433	0.0122	0.1235	0.0172	0.2337

[a-d] Means with different letters in the same column indicate significant differences ($P < 0.05$).

[1] HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.

[2] Analyzed total P in the feeds with and without phytase were in Layer 1: 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.

[3] BWG (0 FYT/kg): $Y = -10.59 + 601.56x$. BWG (4,000 FYT/kg): $Y = 66.91 + 235.35x$. BW: $Y = 1.582 + 0.1344x$. FI: $Y = 109.86 + 7.3825x$. FCR kg/kg: FCR (0 FYT/kg): $Y = 2.0807 - 1.8098x + 2.749x^2$; optimal avP = 0.33%. FCR (4,000 FYT/kg): $Y = 1.8073 - 0.1685x$. FCR kg/dozen eggs: $Y = 1.4737 - 0.8238x + 1.2068x^2$; optimal avP = 0.34%. Egg production (0 FYT/kg): $Y = 78.92 + 70.55x - 96.33x^2$; optimal avP = 0.37%. Egg production (4,000FYT/kg): $Y = 91.1930 + 6.7908x$. Total egg production: $Y = 214.7495 + 29.1682x - 130.6131x^2$; optimal avP = 0.13%.

590 **Table 3**. Quality of eggs from laying hens fed supplemental phytase and graded increases in dietary P from 26 to 57 wks of age.

Phytase, FYT/kg[1]	g	%			kgf	µm
	Egg[3]	Yolk	Albumen	Shell	Breaking strength	Thickness
None	61.4[b]	27.1[a]	63.9	8.94[b]	3.88[b]	378[b]
4,000	61.9[a]	26.8[b]	64.1	9.13[a]	4.05[a]	384[a]
AvP, %[2]						
0.09	60.9	27.2[ab]	63.9	8.95[b]	3.89	378[bc]
0.16	61.7	27.3[a]	63.7	8.99[b]	3.88	378[bc]
0.23	61.6	27.2[ab]	63.9	8.88[b]	3.90	375[c]
0.30	62.1	26.6[c]	64.4	9.08[a]	4.10	384[ab]
0.37	61.9	26.9[bc]	64.0	9.09[a]	4.06	385[a]
0.44	61.6	26.7[c]	64.1	9.21[a]	4.00	387[a]
SEM	0.095	0.049	0.047	0.024	0.029	0.820
Probabilities						
Phytase	0.0032	0.0007	0.2027	<.0001	0.0022	0.0002
AvP linear	0.1314	0.0059	0.2022	0.0012	0.1651	0.0071
AvP quadractic	0.0338	0.5499	0.4938	0.8683	0.3406	0.3580
Phytase*AvP linear	0.9804	0.9341	0.8541	0.7582	0.8967	0.4383
Phytase*AvP quadratic	0.6932	0.5770	0.8869	0.1076	0.8312	0.6863

591 [a-c] Means with different letters in the same column indicate significant differences ($P < 0.05$).
592 [1]HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.
593 [2]Analyzed total P in the feeds with and without phytase were in Layer 1: 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer
594 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.
595 [3] Egg weight: $Y = 59.38 + 14.14x - 23.42x^2$; optimal avP = 0.30%. Yolk percentage: $Y = 28.227 - 1.4933x$. Shell percentage: $Y = 8.396 + 0.7215x$. Shell thickness:
596 $Y = 358.34 + 30.16x$.

Table 4. Apparent ileal digestibility of laying hens fed supplemental phytase and graded increases in dietary P from 26 to 57 wks, dry matter basis[1].

Phytase, FYT/kg[2]	Apparent ileal digestibility[4]					
	%		kcal/kg		%	
	DM	IDEC	IDE	CP	P	Ca
None	65.8	71.6	2837	82.1	48.9[b]	77.2
4,000	68.8	74.3	2960	84.7	72.5[a]	73.5
AvP, %[3]						
0.09	59.4	64.0	2,536	77.9	57.9	85.6[a]
0.16	65.2	73.3	2,873	80.9	68.9	82.2[ab]
0.23	61.2	66.5	2,664	82.2	60.8	74.9[b]
0.30	68.6	74.5	2,971	84.2	57.4	72.5[b]
0.37	72.9	77.0	3,072	84.2	64.1	77.1[ab]
0.44	68.3	72.2	2,916	81.6	61.3	71.9[b]
SEM	1.191	1.302	51.276	1.364	2.159	1.088
Probabilities						
Phytase	0.3789	0.4815	0.4143	0.2720	<.0001	0.2347
AvP linear	0.9039	0.8137	0.7528	0.4377	0.4994	0.0430
AvP quadractic	0.3495	0.7459	0.9316	0.4536	0.9937	0.7946
Phytase*AvP linear	0.0043	0.0152	0.0367	0.0064	0.4034	0.4368
Phytase*AvP quadratic	0.0317	0.1284	0.2608	0.0081	0.9966	0.2109

[a>b] Means with in the same column with different superscripts differ by Tukey test ($p \leq 0.05$).

[1]DM, dry matter; IDCE, ileal digestible energy coefficient; IDE, ileal digestible energy.

[2]HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.

[3]Analyzed total P in the feeds with and without phytase were in Layer 1: 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.

[4] DM digestibility (4,000 FYT/kg): $Y = 68.76 + 57.48x - 273.59x^2$; optimal avP = 0.11%. IDEC (4,000 FYT/kg): $Y = 74.29 + 50.17x$. IDE (4,000 FYT/kg): $Y = 2960.35 + 2017.58x$. CP digestibility (4,000 FYT/kg): $Y = 84.72 + 30.25x - 279.42x^2$; optimal avP = 0.05%. Ca digestibility: $Y = 77.16 - 31.24x$.

606 **Table 5**. Interaction between phytase supplementation and dietary P from 26 to 57 wks of age[1].

Phytase, FYT/kg	AvP	BWG, g/hen	FCR, kg feed/kg egg mass	Hen-day egg production, %	Total eggs, eggs/hen	DM, %	IDEC, %	IDE, kcal/kg	CP, %
0	0.09	-167.6^{c}	1.946^{a}	88.9^{c}	201^{c}	69.3^{a}	73.2^{a}	2,803^{a}	85.5^{a}
	0.16	-37.3^{b}	1.860^{ab}	93.1^{b}	209^{b}	69.3^{a}	75.4^{a}	2,903^{a}	83.1^{a}
	0.23	-41.4^{b}	1.797^{b}	95.4^{ab}	214^{ab}	60.1^{a}	67.5^{a}	2,701^{a}	83.5^{a}
	0.30	14.8^{abc}	1.792^{b}	95.2^{ab}	214^{ab}	67.6^{a}	72.6^{a}	2,886^{a}	82.2^{a}
	0.37	-6.11^{abc}	1.795^{b}	96.3^{a}	218^{a}	68.3^{a}	71.9^{a}	2,855^{a}	80.9^{a}
	0.44	95.5^{a}	1.811^{b}	96.4^{a}	217^{a}	69.1^{a}	72.2^{a}	2,884^{a}	83.3^{a}
4,000	0.09	-48.1^{b}	1.834^{b}	94.7^{ab}	212^{b}	50.8^{b}	56.1^{b}	2,305^{b}	71.4^{b}
	0.16	53.5^{ab}	1.840^{b}	95.2^{ab}	216^{ab}	61.6^{a}	71.5^{a}	2,846^{a}	79.1^{a}
	0.23	22.5^{abc}	1.795^{b}	96.3^{a}	216^{ab}	62.8^{a}	65.6^{a}	2,632^{a}	81.2^{a}
	0.30	90.0^{a}	1.768^{b}	96.6^{a}	218^{a}	69.5^{a}	76.2^{a}	3,045^{a}	85.8^{a}
	0.37	59.6^{ab}	1.796^{b}	97.1^{a}	219^{a}	76.9^{a}	81.5^{a}	3,259^{a}	87.1^{a}
	0.44	49.6^{ab}	1.778^{b}	97.1^{a}	218^{a}	67.7^{a}	72.1^{a}	2,944^{a}	80.1^{a}
SEM		31.877	0.018	0.660	5.718	10.742	12.187	484.821	7.811

607 $^{a>c}$ Means with different letters in the same column indicate significant differences ($P < 0.05$).
608 [1]IDCE, ileal digestible energy coefficient; IDE, ileal digestible energy.
609 [2]HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.
610 [3]Analyzed P with and without phytase were: Layer 1, 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2, 0.35 ± 0.02, 0.41
611 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.

5. ARTIGO II

ESCRITO NAS NORMAS DA REVISTA POULTRY SCIENCE

Supranutritional phytase and dietary phosphorus effects on White Leghorns: II. Bone characteristics, blood biochemical responses, and nutrient utilization

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ABSTRACT

This study evaluated the effects of increasing dietary available phosphorus (avP) concentrations and supranutritional phytase supplementation on bone mineralization, phosphorus (P) utilization, blood biomarkers, and yolk phosvitin concentration in laying hens. A total of 324 Bovans White hens were assigned from 26 to 57 wk of age to a 2 × 6 factorial arrangement consisting of 2 phytase levels (0 or 4,000 FYT/kg) and 6 avP concentrations (0.09, 0.16, 0.23, 0.30, 0.37, and 0.44%). Each treatment included 27 individually housed hens. At 57 wk of age, tibia characteristics, serum biochemical parameters, plasma hormones, yolk phosvitin concentration, and total tract nutrient retention were evaluated. Phytase supplementation increased tibia dry matter (+6.3%), ash (+3.2%), Ca (+5.6%), P (+3.9%), and breaking strength (+16.9%) compared with hens fed feeds without phytase ($P < 0.05$). Increasing avP improved tibia mineralization, although several responses depended on phytase supplementation. Phytase increased serum alkaline phosphatase activity by 46.4%, serum Ca by 26.6%, serum P by 27.0%, plasma parathyroid hormone by 60.1%, and yolk phosvitin concentration by 96.2%, while reducing plasma calcitonin concentration by 6.4% ($P < 0.05$). Serum P increased linearly with increasing avP concentrations ($P < 0.01$). Phytase supplementation also increased P retention by 27.3%, P retention percentage from 19.2 to 26.8%, and apparent metabolizable energy by 234 kcal/kg ($P < 0.05$). Phosphorus retention percentage showed a quadratic response to avP in hens not supplemented with phytase, with an estimated optimum at 0.35% avP, whereas no significant response was observed in phytase-supplemented hens. Overall, supplementation with 4,000 FYT/kg phytase enhanced P utilization, improved bone mineralization, altered physiological indicators of P metabolism, increased yolk phosvitin deposition, and improved energy utilization in laying hens from 26 to 57 wk of age.

Key words: phosphorous, retention, tibia mineralization, biochemical markers

INTRODUCTION

Phosphorus (P) is an essential nutrient for animal growth and development with a primary role in skeletal mineralization, but also essential in cell metabolism and egg production (Leeson and Summers, 2001; Suttle, 2022). Approximately 80 to 85% of total body P is incorporated into bone as hydroxyapatite, contributing to skeletal integrity while functioning as a dynamic mineral reserve that can be mobilized to support physiological requirements (Bonjour, 2011; Serna and Bergwitz, 2020). The continuous requirement for Ca and P during egg production places substantial pressure on mineral homeostasis, as both minerals are continuously mobilized and replenished to support eggshell formation, making adequate dietary supply essential for maintaining productivity and skeletal integrity throughout the laying period (Sinclair-Black et al., 2023).

Corn and soybean meal laying hen feeds contain reasonable amounts of P; however, most of it is bound to poorly utilized phytic acid, as well known (Nelson, 1976; Selle and Ravindran, 2007). Phytase has become a widely adopted nutritional strategy to hydrolyze phytate, increasing P availability and reducing the need for inorganic phosphate use in the feeds (Jlali et al., 2023; Ren et al., 2023). Additionally, phytase has been associated with enhanced trace mineral retention, bone mineralization, and overall energy and nutrient utilization (Jing et al., 2021; Feijó et al., 2023; Moradi et al., 2023; Maria et al., 2024). Contemporary studies have demonstrated that phytase inclusion at levels substantially above conventional recommendations (>1,000 FTU/kg) can further increase phytate degradation and P release, resulting in additional improvements in nutrient utilization and bird performance (Gautier et al., 2018; Ren et al., 2020).

Sustaining egg production while preserving skeletal integrity requires the continuous coordination of Ca and P metabolism, with these minerals dynamically partitioned among the intestine, skeleton, and reproductive tract. Consequently, P homeostasis is maintained through a complex network of physiological and endocrine mechanisms that regulate mineral absorption, retention, mobilization, and deposition according to the bird's metabolic demands (Sommerville and Fox, 1987; Sinclair-Black et al., 2023; Garcia-Mejia et al., 2024). One important destination of dietary P is phosvitin, the main phosphoprotein in the egg yolk. Despite functioning as the primary phosphorus reserve for embryonic development, phosvitin has received relatively little attention in studies of commercial egg production (Mecham and Olcott, 1949; Yamamoto et al., 1997). Although table eggs are not intended to support embryonic development, the mechanisms regulating phosvitin deposition into the yolk remain fundamentally the same, making commercial laying hens a valuable model for investigating phosphorus allocation to the egg. Moreover, understanding these mechanisms has direct relevance to broiler breeder and layer breeder flocks, in which yolk phosvitin constitutes a critical phosphorus source for the developing embryo. Consequently, phosvitin concentration may serve as a useful indicator of dietary P availability, utilization, and partitioning in laying hens (Li et al., 2014).

The objective of the present study was to evaluate the effects of increasing dietary P in feeds supplemented or not with a supranutritional dose of phytase (4,000 FYT/kg) on the metabolic responses of laying hens from 26 to 57 weeks of age. It is hypothesized that phytase used as a supranutritional supplement leads to effects that extend beyond simply satisfying the hen's P requirement, thereby improving the physiological adaptation of hens to increasing dietary P supply and resulting in measurable changes in bone mineralization, phosphorus homeostasis, and P deposition into the egg.

MATERIALS AND METHODS

All procedures utilized in the present study were approved by the Ethics and Research Committee of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil (approval number 46471).

Birds Husbandry and Dietary Treatments

The trial was conducted with 324 Bovans White laying hens obtained at 13 wk of age from a commercial farm (Mercoaves Comércio de Aves Ltda, Bom Princípio, RS, Brazil). Birds were housed individually in electrostatically painted cages (0.33 × 0.46 × 0.40 m), with body weight evenly distributed across treatments. Photoperiodic stimulation started at arrival with a 13L:11D cycle, followed by weekly increases of 30 min until 16L:8D was reached at 19 wks of age and maintained until the end of the trial. Environmental temperature was controlled according to Bovans White management guidelines (Hendrix, 2020).

From 26 wk of age, hens were randomly allocated to 12 dietary treatments arranged in a 2 × 6 factorial, with two levels of phytase supplementation (0 or 4,000 FYT/kg) and six concentrations of avP (0.09, 0.16, 0.23, 0.30, 0.37, and 0.44%, which corresponded to 0.07, 0.12, 0.17, 0.22, 0.26 and 0.31% digestible P (dP)). Each treatment included 27 replicates with a single hen per cage. The lowest P level was achieved by omitting supplemental phosphate, while graded increments (0.07% units) were obtained by adding dicalcium phosphate up to the value recommended in the strain manual.

Feeds were based on corn and soybean meal and formulated according to commercial standards for each production phase: Grower II (13–16 wk), Pre-Lay (17–20 wk), Layer I (Table 1; 21–37 wk), and Layer II (Table 1; 38–57 wk). Representative samples of each feed were collected from 140-kg batches manufactured every 28 days and stored at −20 °C until

118 analysis. Analyzed total P contents of the experimental feeds were 0.34% ± 0.02, 0.41% ±
119 0.03, 0.49% ± 0.02, 0.55% ± 0.03, 0.61% ± 0.06, and 0.69% ± 0.04.

120 Phytase was included as a coated 6-phytase product (Ronozyme HiPhorius, 40,000
121 FYT/g; Novonesis A/S, Bagsvaerd, Denmark) at 100 g/ton to achieve 4,000 FYT/kg
122 (analyzed activity: 4,186 ± 432 FYT/kg). The dose, higher than usual commercial
123 recommendations, was chosen to maximize phytate hydrolysis in corn and soybean meal
124 without attributing extra Ca or P values in the formulation. During the last 4 wk of the study
125 (54–57 wk), 1% Celite (Celite Corp., Lompoc, CA) was included in all feeds as an
126 indigestible marker.

127 ***Collection of Excreta and Eggs***

128 During the last three days of the trial (wk 57), total excreta and eggs were collected
129 from 10 hens per treatment. Excreta were collected twice daily on wax paper, immediately
130 homogenized, pooled by cage, and stored at −20 °C until analysis. Eggs produced by these
131 hens during the same period were also collected and weighed. On the last day of the
132 experiment, 10 eggs per treatment were collected for yolk phosvitin analysis. Yolks were
133 separated, homogenized by hand, and stored at −20 °C until analysis. Phosvitin
134 concentrations were determined using a sandwich ELISA kit (Ref: NB-E60011, Novatein
135 Biosciences, Woburn, MA, USA) following the manufacturer's instructions. For the assay,
136 yolk samples were diluted 1:45,000 through a three-step process: (A) 2 g of yolk were mixed
137 with 60 mL of PBS to obtain a 1:30 dilution; (B) 20 µL of dilution A were added to 1 mL of
138 PBS to reach a 1:50 dilution (corresponding to 1:1,500 cumulative dilution); (C) 10 µL of
139 dilution B were mixed with 300 µL of sample buffer to obtain the final 1:45,000 dilution.

140 ***Blood and Tissue Sampling***

Blood samples were collected during wk 57 through wing vein puncture 12 h before the expected oviposition of 10 hens per treatment. Only hens with confirmed daily egg production were sampled to reduce variability associated with laying status, ensuring that the samples reflected active shell calcification (Miller et al., 1977; Yan et al., 2023). Collected blood was partially transferred into 0.5 mL EDTA-coated tubes and analyzed using sandwich ELISA for chicken parathyroid hormone (PTH; LS-F31836-1) and calcitonin (CT; LS-F41837-1) according to the manufacturer's instructions (LS Bio, Newark, CA, USA). Only hens fed the lowest and highest avP feeds (0.09% and 0.44%) were included in the PTH and CT analyses. Remaining blood was centrifuged to obtain serum, and serum levels of alkaline phosphatase (ALP), Ca, and P concentrations were determined by dry chemistry using an automatic analyzer (VITROS 250, Ortho Clinical Diagnostics, Rochester, NY, USA)

On the last day of the trial, tibiae were collected from the same 10 hens per treatment used for blood sampling after euthanasia by cervical dislocation, and the surrounding muscle tissue was removed. Left tibia was dried, ashed, and Ca and P concentrations were determined (AOAC, 2019). Right tibia was used for breaking strength measurements using a texture analyzer (TA.XT. plus, Texture Technologies, Hamilton, MA, USA) according to Shim et al. (2012).

Analyses and Calculations

All samples were oven-dried at 105 °C for 12 h prior to analysis (method 934.01; AOAC International, 2006). Excreta and feed samples were analyzed for gross energy (GE) using a calorimeter calibrated with benzoic acid as a standard (IKA Werke, Parr Instruments, Staufen, Germany). Crude protein (N × 6.25) in feed was determined by the combustion method (method 968.06; AOAC International, 2006). Ca and P concentrations were measured in feed, excreta, and whole eggs according to AOAC (2019), whereas acid-

insoluble ash was determined only in feed and excreta following Vogtmann et al. (1975) and Choct and Annison (1992). Total tract retention of Ca, P, and apparent metabolizable energy corrected for nitrogen retention (AMEn) was calculated based on the equations of Kong and Adeola (2014), considering excretion values from both eggs produced and total excreta collected. All calculations were based on analyzed values expressed on a dry matter basis.

Statistical Analysis

All data were primarily tested for normality and homogeneity of variances using the Shapiro–Wilk and Levene tests, respectively (Levene, 1960; Shapiro and Wilk, 1965). Data were transformed using the arcsine square root transformation (z = asin (sqrt (y + 0.5))) whenever not normally distributed (Ahrens et al., 1990). Analyses of variance were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA), and significance was declared at $P \leq 0.05$. Data were analyzed as a completely randomized design using the GLM procedure of SAS. Phytase was considered a fixed qualitative factor and avP a quantitative factor. The cage was considered the experimental unit.

Phytase avP equivalency was estimated by substituting the observed response of hens fed 0.09% avP supplemented with phytase into the regression equation obtained for hens fed diets without phytase, using yolk phosvitin concentration, P retention (%), and tibia P concentration as response criteria. Linear (L) and quadratic (Q) effects of avP were evaluated. When significant effects were detected, and when applicable, the dietary avP that maximized the responses was estimated using the x-coordinate of the vertex of the quadratic equations ($x = -b/2a$). Mean separation was performed using Tukey's test (Tukey, 1991) when significant effects were observed. Experimental diets were formulated on an avP basis, which served as the treatment definition and statistical analyses. However, because digestible phosphorus (dP) is increasingly being adopted as a more physiologically relevant and modern

system for P evaluation and feed formulation, the corresponding dP values are also presented to facilitate interpretation and comparison across feeding systems.

RESULTS

Results for tibia characteristics are presented in Table 2. Phytase × avP interactions were observed for tibia ash (linear; P = 0.0146), Ca (linear; P = 0.0076), P (linear; P = 0.0018), and breaking strength (quadratic; P = 0.0037).

Tibia ash concentration increased linearly with increasing avP in hens fed diets without phytase (Y = 47.9456 + 20.7989x; P < 0.0001) and with phytase supplementation (Y = 48.9336 + 8.2331x; P = 0.0188). In contrast, tibia Ca and P concentrations increased linearly only in hens fed diets without phytase (Ca: Y = 16.4203 + 7.1617x; P < 0.0001; P: Y = 8.3021 + 3.6935x; P < 0.0001), with no significant regressions observed in phytase-supplemented hens. Similarly, tibia breaking strength responded differently according to phytase supplementation, showing a quadratic response in hens fed diets without phytase (Y = 11.6905 + 8.6586x + 86.9142x^2; P = 0.0086), with the lowest estimated value at 0.22% avP, whereas a linear increase was observed in phytase-supplemented hens (Y = 16.1383 + 19.9688x; P < 0.0001).

Supplementation with 4,000 FYT/kg phytase increased tibia dry matter (DM; +6.3%; P = 0.0008), ash (+3.2%; P = 0.0148), Ca (+5.6%; P = 0.0015), and P (+3.9%; P = 0.0027) concentrations, as well as tibia breaking strength (+16.9%; P < 0.0001). Independently, increasing dietary avP linearly increased tibia DM (Y = 50.6500 + 24.0776x; P < 0.0001).

Results for blood biochemical parameters and yolk phosvitin concentration are presented in Table 3. Phytase × avP interactions were observed for serum ALP activity (linear; P = 0.0246) and yolk phosvitin concentration (quadratic; P < 0.0001). Serum ALP activity decreased linearly with increasing avP in hens fed feeds without phytase (Y =

673.0497 − 864.3111x; P = 0.0113) and with phytase supplementation (Y = 894.8542 − 2340.9524x; P < 0.0001). Overall, phytase increased serum ALP activity by 46.4% (P < 0.0001). Yolk phosvitin concentration showed quadratic responses to avP both in hens fed feeds without phytase ($Y = 4070.8120 + 14835.3055x + 49712.8002x^2$; P = 0.0025), with the lowest estimated value occurring at 0.12% avP, and in phytase-supplemented hens ($Y = 11754.4105 + 16592.4975x - 136545.0039x^2$; P < 0.0001), with an estimated optimum at 0.33% avP. Overall, phytase increased yolk phosvitin concentration by 96.2% (P < 0.0001).

Overall, phytase supplementation increased serum P concentration by 27% (6.75 vs. 8.57 mg/dL; P = 0.0139). Independently, serum P increased linearly (P = 0.0018) with increasing dietary avP according to the equation Y = 7.6734 + 6.3229x (P = 0.0012). Serum Ca concentration, plasma PTH, and plasma calcitonin were affected only by phytase supplementation. Phytase increased serum Ca concentration by 26.6% (P = 0.0147) and plasma PTH by 60.1% (P = 0.0013), whereas plasma calcitonin concentration was reduced by 6.4% (P = 0.0406). Dietary avP did not affect serum Ca, plasma PTH, or plasma calcitonin concentrations (P > 0.05).

Results for total tract retention responses are presented in Table 4. Phytase × avP interactions were observed for P excretion (linear; P = 0.0140), P retention (g/day; linear; P = 0.0260), P retention percentage (linear; P = 0.0009), and AMEn (quadratic; P = 0.0378). Phosphorus excretion increased linearly in hens fed feeds without phytase (Y = 0.3754 + 0.6607x; P < 0.0001) and with phytase supplementation (Y = 0.3794 + 0.9839x; P < 0.0001), corresponding to estimated increases of 0.046 and 0.069 g/day, respectively, for each 0.07 percentage-point increase in dietary avP. Overall, phytase did not affect P excretion (P = 0.8634). Likewise, P retention (g/day) increased linearly in hens fed feeds without phytase (Y = 0.1255 + 0.5393x; P < 0.0001) and with phytase supplementation (Y = 0.1502 +

0.2470x; P = 0.0152), with estimated increases of 0.038 and 0.017 g/day, respectively. Overall, phytase increased P retention by 27.3% (0.11 vs. 0.14 g/day; P = 0.0165).

Although P retention percentage was affected quadratically by dietary avP in the overall model (P = 0.0163), a significant linear phytase × avP interaction was detected (P = 0.0009). Therefore, regressions were evaluated separately for each phytase level. In hens fed without phytase, P retention percentage showed a quadratic response to increasing avP levels ($Y = 25.0023 + 76.7808x - 436.0622x^2$; P = 0.0203), with an estimated maxima at 0.35% avP. In contrast, no significant regression was observed in hens supplemented with 4,000 FTU/kg phytase (P = 0.5950). Overall, phytase increased P retention percentage from 19.2 to 26.8% (P = 0.0115). Apparent metabolizable energy showed different responses depending on phytase supplementation. In hens fed without phytase, AMEn showed a quadratic response ($Y = 3165.55 + 1443.21x - 6750.53x^2$; P = 0.0120), with an estimated maxima at 0.37% avP, whereas in phytase fed hens AMEn increased linearly (Y = 3309.24 + 503.28x; P = 0.0043) at 35.2 kcal/kg per avP increment. Overall, phytase increased AMEn by 234 kcal/kg (P = 0.0129).

Dry matter content, Ca intake, Ca excretion, Ca retention (%), and P intake were independently affected by phytase and/or dietary avP. Phytase supplementation increased DM content by 5.9% (P = 0.0097), Ca intake by 10.7% (P = 0.0008), P intake by 8.2% (P = 0.0011), and Ca retention (g/day) (P = 0.0484). Dry matter increased linearly with dietary avP as Y = 69.6654 + 24.8526x (P < 0.0001). Although a quadratic effect of dietary avP was detected in the overall model (P = 0.0425), the quadratic term of the fitted regression equation was not significant (P = 0.0882). Therefore, Ca intake was best described by a linear regression (Y = 3.93228 + 3.00516x; P < 0.0001).

Calcium excretion increased linearly according to the equation $Y = 2.3943 + 4.2194x$ ($P < 0.0001$), corresponding to an estimated increase of 0.295 g/day for each 0.07 percentage-point increase in dietary avP. In contrast, Ca retention percentage decreased linearly ($Y = 40.2642 - 66.3680x$; $P < 0.0001$), declining by 4.65 percentage units across successive dietary avP levels. Phosphorus intake also increased linearly with dietary avP ($Y = 0.5077 + 1.2061x$; $P < 0.0001$), corresponding to an estimated increase of 0.084 g/day for each 0.07 percentage-point increase in dietary avP.

DISCUSSION

The present study demonstrates that dietary phytase supplementation at 4,000 FYT/kg markedly enhances P utilization, skeletal integrity, mineral retention, and energy efficiency in laying hens with these responses being strongly modulated by dietary avP level.

Bones represent important mineral storage, and tibia characteristics are commonly used as indicators of skeletal mineralization and structural integrity in laying hens (Rath et al., 2000; Whitehead and Fleming, 2000; Olgun and Aygun, 2016). The responses observed in the present study indicate that both phytase supplementation and dietary P supply influenced bone mineralization and skeletal integrity. The improvement in tibia characteristics associated with phytase supplementation is likely related to the enzyme's ability to hydrolyze phytate, releasing P that would otherwise remain unavailable to the bird, thereby increasing intestinal P absorption and its subsequent deposition in bone tissue (Selle and Ravindran, 2007; Humer et al., 2015). Likewise, the effects of dietary P supply on tibia characteristics demonstrate the close relationship between P availability and skeletal mineralization. Importantly, these findings reinforce that bone tissue is a sensitive indicator of changes in P availability, often responding more consistently than circulating mineral concentrations.

The observed responses in tibia characteristics are consistent with the central role of P in bone formation and maintenance. The study findings agree with previous ones demonstrating that phytase improves bone mineralization and structural integrity in laying hens by increasing P availability and its incorporation into skeletal tissue (Mansoori and Modirsanei, 2015; Bello et al., 2020; Farias et al., 2021; Rath et al., 2000; Li et al., 2017).

Phosvitin is the most highly phosphorylated protein found in nature and represents the main P storage protein in egg yolk, playing an important role in mineral binding and antioxidant protection in the developing embryo (Mecham and Olcott, 1949; Burley and Cook, 1961; Samaraweera et al., 2011). The high number of phosphorylated serine residues in phosvitin confers a strong capacity to bind minerals, particularly iron and other divalent cations, which contributes to its biological functions (Taborsky, 1963; Samaraweera et al., 2011). The results of the present study suggest that phosvitin deposition in egg yolk may be influenced by dietary P supply and its availability (Burley and Cook, 1961; Samaraweera et al., 2011; Yilmaz and Agagunduz, 2020). These observations support the notion that improvements in P utilization promoted by phytase may be reflected not only in skeletal tissue but also in the deposition of P-containing compounds in the egg yolk.

An interesting aspect of the present study was that phytase supplementation shifted the estimated dietary P concentration associated with maximum yolk phosvitin deposition to higher values (0.12 to 0.33% of avP) and this response reflects the greater availability of absorbable P generated by phytate hydrolysis rather than an increase in the hens' P requirement. Under phytase supplementation, additional P released from phytate (Cowieson et al., 2009; Adeola and Cowieson, 2011) may have remained available for deposition into the yolk across a broader range of dietary P concentrations, resulting in a flatter response curve and consequently a higher estimated optimum. These shifts should be interpreted

cautiously because quadratic estimates are strongly influenced by the shape of the fitted response rather than solely by biological requirements (Pesti et al., 2009).

The mineral metabolism in laying hens is tightly regulated by endocrine mechanisms that control Ca and P homeostasis in order to maintain skeletal integrity and support eggshell formation (Li et al., 2017; Proszkowiec-Weglarz and Angel, 2013, Sinclair-Black et al., 2023). Among the biochemical markers evaluated in the present study, ALP is known to be important in bone metabolism because of its association with osteoblastic activity and mineral deposition during bone formation (Golub and Boesze-Battaglia, 2007; Vimalraj, 2020). In the present study, phytase supplementation significantly increased serum ALP activity, indicating greater metabolic activity associated with bone mineralization, which may be attributed to the increased availability of P released from phytate and its enhanced absorption and utilization (Selle and Ravindran, 2007; Humer et al., 2015). In contrast, serum ALP activity decreased linearly with increasing dietary avP, indicating that greater P availability reduces bone turnover and the metabolic demand for osteoblastic activity, whereas lower P levels may stimulate bone remodeling to maintain mineral homeostasis, as previously reported (Zhou et al., 2009; Nakagi et al., 2013; Lim et al., 2020). Collectively, these findings indicate that the effects of phytase are not equivalent to simply increasing dietary P levels, but instead involve coordinated physiological adjustments in bone turnover and mineral metabolism, particularly under conditions of limited P availability.

Serum Ca and P concentrations are important indicators of mineral metabolism in laying hens (Cheng et al., 2025). These minerals are essential components of hydroxyapatite crystals that provide rigidity and mechanical strength to bone tissue and are also required for eggshell formation (Rath et al., 2000; Whitehead and Fleming, 2000). Changes in circulating Ca and P concentrations may therefore provide insight into the efficiency of mineral

absorption and utilization. Previous studies have shown that phytase supplementation can increase the availability and circulating concentrations of Ca and P through enhanced phytate hydrolysis and improved mineral digestibility (Musapuor et al., 2005; Lim et al., 2020; Saleh et al., 2021). Likewise, serum P concentration is considered a sensitive indicator of dietary P supply, generally increasing in response to greater P intake and availability (Boorman and Gunaratne, 2001).

Parathyroid hormone is a major regulator of Ca and P homeostasis, acting to maintain circulating mineral concentrations through mechanisms that involve bone mobilization and renal regulation of mineral reabsorption (Wideman, 1987; Proszkowiec-Weglarz and Angel, 2013; Li et al., 2017). In the present study, phytase supplementation increased plasma PTH concentration. Calcitonin, on the other hand, acts as a hormone that inhibits bone resorption and contributes to the regulation of Ca homeostasis (Pondel, 2001; Li et al., 2017; Shaker and Deftos, 2023). In contrast to the increase observed for PTH, phytase supplementation reduced plasma calcitonin concentration. This reduction may be associated with changes in endocrine regulation resulting from improved mineral availability.

The opposite responses of PTH and calcitonin suggest a shift in hormonal balance toward enhanced mineral turnover and redistribution, likely reflecting adjustments to increased P availability. The balance between PTH and CT plays an important role in maintaining mineral homeostasis, and alterations in these hormones may reflect adjustments in bone metabolism and mineral utilization in response to dietary treatments although PTH appears to be more responsive to dietary P supply than calcitonin. (Felsenfeld and Levine, 2015; Ren et al., 2023; Shaker and Deftos, 2023).

Phytase supplementation improved P utilization primarily by increasing the hydrolysis of phytate and releasing phytate-bound P, thereby increasing the amount of

absorbable P available to the birds (Selle and Ravindran, 2007). Beyond its direct effect on P availability, phytase also contributes to improving the utilization of other nutrients by reducing the antinutritional properties of phytate, which can interfere with the digestion and absorption of proteins, amino acids, and energy (Manangi et al., 2009; Walk and Olukosi, 2019). Consequently, improvements in AMEn are expected when phytase is included in layer feeds, reflecting a broader enhancement of digestive efficiency rather than solely increased P release. isms were reflected by the simultaneous increase in AMEn and P retention in phytase-supplemented hens, indicating that the greater availability of phytate-derived P was accompanied by improved overall nutrient utilization. Previous studies in laying hens and broilers consistently demonstrate that phytase increases P retention while reducing the need for inorganic P supplementation (Gordon and Roland, 1997; Boling et al., 2000; Ravindran et al., 2000).

The responses to increasing dietary avP indicate that mineral utilization is tightly regulated according to the hens' physiological requirements. Although a greater dietary P supply inevitably increases mineral intake, the efficiency with which absorbed minerals are retained declines once the metabolic demand for growth, maintenance, and eggshell formation is satisfied (Leske and Coon, 2002). Accordingly, increasing dietary avP progressively increased both Ca and P intake, whereas Ca retention remained relatively constant and its retention efficiency declined, suggesting that additional Ca intake was not matched by greater physiological demand. In contrast, P retention increased with increasing avP supply, but P excretion also increased substantially, indicating that once tissue and egg production requirements were progressively met, a larger proportion of the additional dietary P was eliminated rather than retained. Such responses are consistent with previous reports

379 describing reduced mineral utilization efficiency as dietary P supply exceeds the birds'
380 requirements (Ravindran et al., 2000; Shastak et al., 2012).

381 The magnitude of the compensatory effect of supranutritional phytase can also be
382 illustrated by its estimated avP equivalency based on phosphorus metabolism responses.
383 Using yolk phosvitin concentration as the response criterion, hens fed 0.09% avP
384 supplemented with phytase achieved a response comparable to that of hens fed approximately
385 0.22% avP without phytase, corresponding to an avP equivalency of about 0.13 percentage
386 units. Similarly, phytase supplementation was equivalent to approximately 0.16 percentage
387 units of avP based on both phosphorus retention (%) and tibia phosphorus concentration.
388 These findings demonstrate that phytase effectively compensated for dietary P deficiency
389 under the most P-limiting conditions.

390 **CONCLUSIONS**

391 Supranutritional phytase supplementation (4,000 FYT/kg), included as a research
392 model to maximize phytate degradation and investigate the physiological consequences of
393 enhanced P availability rather than to represent a commercially recommended dose, altered
394 the physiological responses of laying hens to dietary P by improving P use efficiency and
395 modulating mineral metabolism, skeletal mineralization, nutrient utilization, and P
396 deposition into the egg. Under the conditions of the present study, dietary avP concentrations
397 between 0.35 and 0.37% were sufficient to optimize most responses in hens fed diets without
398 phytase, whereas phytase supplementation provided an estimated avP equivalency ranging
399 from 0.13 to 0.16 percentage units, suggesting that dietary avP concentrations between
400 approximately 0.19 and 0.24% may be sufficient to maintain phosphorus utilization and
401 mineralization responses while reducing the reliance on inorganic phosphate
402 supplementation in laying hen feeds.

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Table 1. Ingredient and nutrient composition of feeds having supplemental phytase and graded dietary P increases.

Ingredients, % as-is	Layer I (21 to 37)						Layer II (38 to 57)					
	0.09	0.16	0.23	0.30	0.37	0.44	0.09	0.16	0.23	0.30	0.37	0.44
Corn	55.0	54.8	54.6	54.4	54.3	54.1	55.7	55.4	55.3	55.1	54.9	54.7
Soybean meal, 46%	28.7	28.7	28.7	28.8	28.8	28.8	27.6	27.7	27.7	27.7	27.8	27.8
Soybean oil	3.1	3.1	3.2	3.3	3.3	3.4	3.06	3.12	3.19	3.26	3.32	3.39
Dicalcium phosphate	-	0.40	0.80	1.10	1.50	1.8	-	0.34	0.67	1.11	1.44	1.77
Limestone	12.1	11.9	11.6	11.3	11.0	10.8	12.4	12.2	11.9	11.6	11.3	11.1
Salt	0.34						0.37					
Methionine analogue[1]	0.29						0.26					
L-Lysine HCL	0.01						0.01					
L-Threonine	0.03						0.11					
Vitamin and minerals[2]	0.35						0.35					
Choline chloride	0.12						0.13					
Total	100.00						100.00					
AMEn, kcal/kg	2,860						2,860					
Crude protein	17.35						17.00					
Ca	4.1						4.2					
AvP	0.09	0.16	0.23	0.30	0.37	0.44	0.09	0.16	0.23	0.30	0.37	0.44
Dig. P	0.07	0.12	0.17	0.22	0.26	0.31	0.07	0.12	0.17	0.22	0.26	0.31
NPP	0.08	0.14	0.21	0.28	0.34	0.41	0.08	0.14	0.21	0.28	0.34	0.41
Total P[4]	0.29	0.35	0.42	0.49	0.55	0.62	0.29	0.35	0.42	0.49	0.55	0.62
Na	0.17						0.18					
d Lys	0.85						0.83					
d Met	0.50						0.47					
d TSAA	0.76						0.72					
d Trp	0.149						0.19					
d Thr	0.60						0.66					
d Val	0.75						0.73					
Choline, mg/kg	1,700						1,700					
Phytate[5]	0.21						0.20					
Phytase, FYT/kg[6]	4,208±482						4,164±308					

[1] Novus (DL-2-hydroxy-(4-methylthio) butanoic acid).

[2] Composition per kg of product: vitamin A, 4,250,000 IU; vitamin D3, 1,650,000 IU; vitamin E, 10,000 IU; vitamin K3, 1,500 mg; vitamin B1, 1,000 mg; vitamin B2, 2,300 mg; vitamin B6, 1,500 mg; pantothenic acid, 6 g; vitamin B12, 12,500 mcg; nicotinic acid, 20 g; folic acid, 400 mg; biotin, 100 mg; Se, 130 mg; Fe, 22.5 g; Mn, 40 g; Zn, 27.5 g; I, 500 mg; Cu, 3,250 mg; butylated hydroxytoluene, 28.6 g; Rovabio Advance (Adisseo France S.A.S., Antony, France), 142.9 g.

[3]Analyzed total P in the feeds with and without phytase: Layer 1, 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.

[4]Phytate values using NIRS

[5]HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.

Table 2. Tibiae analysis of laying hens fed supplemental phytase and graded increases in dietary P at 57 wks, dry matter basis.

Phytase, FYT/kg[1]	%				kgf
	DM[3]	Ash	Ca	P	Breaking strength
None	49.1	47.1	16.2	8.16	13.0
4,000	52.2	48.6	17.1	8.48	15.2
AvP, %[2]					
0.09	45.6^{d}	44.5^{c}	15.7^{c}	7.80^{c}	12.1^{b}
0.16	47.9^{cd}	46.3^{bc}	16.0^{bc}	8.06^{bc}	11.9^{b}
0.23	50.6^{bc}	49.1^{ab}	16.8^{abc}	8.48^{ab}	13.8^{ab}
0.30	53.7^{ab}	48.1^{ab}	16.7^{abc}	8.38^{ab}	15.0^{ab}
0.37	51.6^{abc}	49.0^{ab}	16.9^{ab}	8.49^{ab}	14.9^{ab}
0.44	54.6^{a}	50.1^{a}	17.4^{a}	8.75^{a}	16.9^{a}
SEM	0.482	0.355	0.139	0.062	0.490
Probabilities					
Phytase	0.0008	0.0148	0.0015	0.0027	<.0001
AvP linear	<.0001	<.0001	<.0001	<.0001	0.0239
AvP quadractic	0.5274	0.1031	0.2615	0.1105	0.0214
Phytase*AvP linear	0.3574	0.0146	0.0076	0.0018	0.0399
Phytase*AvP quadratic	0.3621	0.4696	0.2509	0.1587	0.0037

[a-d] Means with different letters in the same column indicate significant differences ($P < 0.05$).

[1] HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.

[2]Analyzed total P in the feeds with and without phytase were in Layer 1: 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.

[3] DM: $Y = 50.6500 + 24.0776x$. Ash (0 FYT/kg): $Y = 47.9456 + 20.7989x$. Ash (4,000 FYT/kg): $Y = 48.9336 + 8.2331x$. Ca (0 FYT/kg): $Y = 16.4203 + 7.1617x$. P (0 FYT/kg): $Y = 8.3021 + 3.6935x$. Breaking strength (0 FYT/kg): $Y = 11.6905 + 8.6586x + 86.9142x^2$; optimal avP = 0.22%. Breaking strength (4,000 FYT/kg): $Y = 16.1383 + 19.9688x$.

628 **Table 3**. Biochemical parameters of laying hens fed supplemental phytase and graded
629 increases in dietary P at 57 wks.

Phytase, FYT/kg[1]	U/L	mg/dL		pg/ml		µg/ml
	ALP[3]	Ca	P	PTH	CT	Phosvitin
None	692	22.9	6.75	134.8	305.3	4,960
4,000	1,013	29.0	8.57	215.8	285.9	9,733
Av. P, %[2]						
0.09	1,143[a]	21.9	5.86[b]	148.7	308.1	2,834[b]
0.16	1,137[a]	25.5	7.64[ab]	-	-	7,766[a]
0.23	880[ab]	24.8	7.74[ab]	-	-	7,619[a]
0.30	601[b]	33.2	7.96[ab]	-	-	7,419[a]
0.37	688[b]	25.5	8.01[ab]	-	-	8,697[a]
0.44	664[b]	25.0	8.73[a]	201.8	283.2	9,939[a]
SEM	46.310	1.205	0.242	13.934	13.198	346.592
Probabilities						
Phytase	<.0001	0.0147	0.0139	0.0013	0.0406	<.0001
AvP linear	0.0491	0.7229	0.0018	0.2420	0.3390	<.0001
AvP quadractic	0.8208	0.6050	0.3337	-	-	0.0025
Phytase*AvP linear	0.0246	0.6461	0.3268	0.5254	0.6219	0.4428
Phytase*AvP quadratic	0.2515	0.3031	0.8101	-	-	<.0001

630 [a-b] Means with different letters in the same column indicate significant differences ($P < 0.05$).
631 [1] HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.
632 [2]Analyzed total P in the feeds with and without phytase were in Layer 1: 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02,
633 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02, 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ±
634 0.03 and 0.69 ± 0.03 %.
635 [3] ALP (0 FYT/kg): $Y = 673.0497 - 864.3111x$. ALP (4,000 FYT/kg): $Y = 894.8542 - 2340.9524x$. P: $Y =$
636 $7.6734 + 6.3229x$. Phosvitin (0 FYT/kg): $Y = 4070.8120 + 14835.3055x + 49712.8002x^2$; optimal avP = 0.12%.
637 Phosvitin (4,000 FYT/kg): $Y = 11754.4105 + 16592.4975x - 136545.0039x^2$; optimal avP = 0.33%.

638 **Table 4**. Tract retention responses of laying hens fed supplemental phytase and graded increases in dietary P at 57 wks.

Phytase, FYT/kg[2]	DM, %[4]	Ca				P				AME, kcal/kg
		Intake	Excretion[1]	Retention	Retention	Intake	Excretion[1]	Retention	Retention	
		g/day			%	g/day			%	
None	67.6	3.73	2.30	1.44	40.0	0.49	0.38	0.11	19.2	3,069
4,000	71.6	4.13	2.48	1.65	40.8	0.53	0.39	0.14	26.8	3,303
Av. P, %[3]										
0.09	62.5^{b}	3.36^{b}	1.65^{b}	1.70	50.6^{ab}	0.29^{d}	0.26^{d}	0.04^{c}	11.5^{b}	2,957^{c}
0.16	69.0^{a}	3.40^{b}	1.59^{b}	1.81	54.8^{a}	0.36^{d}	0.27^{d}	0.09^{bc}	23.2^{ab}	3,107^{bc}
0.23	69.8^{a}	3.99^{ab}	2.45^{a}	1.54	38.5^{bc}	0.49^{c}	0.36^{c}	0.13^{ab}	25.7^{ab}	3,212^{ab}
0.30	70.6^{a}	4.33^{a}	2.94^{a}	1.39	32.4^{c}	0.56^{b}	0.41^{bc}	0.15^{ab}	26.1^{a}	3,242^{ab}
0.37	73.5^{a}	4.28^{a}	2.78^{a}	1.45	34.5^{c}	0.63^{b}	0.46^{ab}	0.16^{ab}	25.6^{ab}	3,294^{a}
0.44	72.0^{a}	4.29^{a}	2.92^{a}	1.37	31.6^{c}	0.71^{a}	0.53^{a}	0.18^{a}	25.1^{ab}	3,303^{a}
SEM	0.647	0.072	0.083	0.054	1.546	0.017	0.013	0.009	1.738	25.941
Probabilities										
Phytase	0.0097	0.0008	0.2136	0.0484	0.6086	0.0011	0.8634	0.0165	0.0115	0.0129
AvP linear	<.0001	<.0001	<.0001	0.1857	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
AvP quadractic	0.0766	0.0425	0.1729	0.5259	0.7225	0.1360	0.8770	0.1139	0.0163	0.0025
Phytase*AvP linear	0.2290	0.6239	0.8495	0.4403	0.9447	0.8057	0.0140	0.0260	0.0009	0.0027
Phytase*AvP quadratic	0.8590	0.4346	0.7763	0.2302	0.4038	0.2335	0.6565	0.4834	0.2352	0.0378

639 [a-d] Means with different letters in the same column indicate significant differences ($P < 0.05$);

640 [1] Sum of total excreta and eggs mineral content.

641 [2] HiPhorius 40,000 FYT/g, Novonesis A/S, Bagsvaerd, Denmark.

642 [3]Analyzed total P in the feeds with and without phytase were in Layer 1: 0.34 ± 0.02, 0.41 ± 0.02, 0.49 ± 0.02, 0.55 ± 0.02, 0.62 ± 0.03 and 0.69 ± 0.04 %; Layer 2: 0.35 ± 0.02,
643 0.41 ± 0.03, 0.48 ± 0.03, 0.55 ± 0.03, 0.63 ± 0.03 and 0.69 ± 0.03 %.

644 [4] DM: $Y = 69.6654 + 24.8526x$. Ca intake: $Y = 3.93228 + 3.00516x$. Ca excretion: $Y = 2.3943 + 4.2194x$. Ca retention (%): $Y = 40.2642 - 66.3680x$. P intake: ($Y = 0.5077 +$
645 $1.2061x$. P excretion (0 FYT/kg): $Y = 0.3754 + 0.6607x$. P excretion (4,000 FYT/kg): $Y = 0.3794 + 0.9839x$. P retention (g/day) (0 FYT/kg): $Y = 0.1255 + 0.5393x$. P retention
646 (g/day) (4,000 FYT/kg): $Y = 0.1502 + 0.2470x$. P retention (%) (0 FYT/kg): $Y = 25.0023 + 76.7808x - 436.0622x^2$; optimal avP = 0.35%. AME (0 FYT/kg): $Y = 3165.55 +$
647 $1443.21x - 6750.53x^2$; optimal avP = 0.37%. AME (4,000 FYT/kg): $Y = 3309.24 + 503.28x$.

6. CONSIDERAÇÕES FINAIS

Os resultados desta tese demonstraram que a suplementação de fitase em dose supranutricional (4.000 FYT/kg) modifica significativamente as respostas de poedeiras comerciais aos níveis dietéticos de fósforo, aumentando a eficiência de utilização desse nutriente e reduzindo a dependência de fontes inorgânicas de suplementação. A presença de interações entre fitase e fósforo disponível evidenciou que a enzima foi particularmente eficiente em minimizar os efeitos da deficiência fosfórica, permitindo manter o desempenho produtivo e a qualidade da casca dos ovos mesmo em dietas com menores concentrações de fósforo.

Na ausência de fitase, concentrações de aproximadamente 0,35 a 0,37% de fósforo disponível foram necessárias para otimizar a maioria das respostas produtivas e fisiológicas avaliadas. Em contraste, a suplementação com 4.000 FYT/kg de fitase proporcionou uma equivalência de aproximadamente 0,10 a 0,12 pontos percentuais de fósforo disponível, permitindo reduzir os níveis dietéticos para cerca de 0,23 a 0,27% sem prejuízos ao desempenho das aves.

Além dos efeitos sobre desempenho e qualidade dos ovos, a suplementação de fitase alterou importantes respostas fisiológicas relacionadas ao metabolismo mineral, promovendo melhorias na utilização e retenção do fósforo, na mineralização óssea, na digestibilidade dos nutrientes e na deposição de fósforo no ovo. Os resultados obtidos reforçam que a avaliação das exigências de fósforo em poedeiras deve considerar não apenas parâmetros produtivos, mas também indicadores ósseos e metabólicos capazes de refletir a eficiência de utilização desse nutriente pelo organismo.

De forma geral, esta tese demonstra que a utilização de fitase em dose supranutricional constitui uma estratégia eficaz para aumentar o aproveitamento do fósforo presente nos ingredientes vegetais, contribuindo para a redução da inclusão de fosfatos inorgânicos, para a melhoria da eficiência produtiva e para o desenvolvimento de sistemas de produção de ovos mais sustentáveis.

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Papers must be written in English. The text and all supporting materials must use American spelling and usage as given in The American Heritage Dictionary, Webster's Third New International Dictionary, or the Oxford American English Dictionary. Authors should follow the style and form recommended in Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers. 2006. 7th ed. Style Manual Committee, Council of Science Editors, Reston, VA.

Preparing the manuscript file

Manuscripts should be typed double-spaced, with lines and pages numbered consecutively, using Times New Roman font at 12 points. All special characters (e.g., Greek, math, symbols) should be inserted using the symbols palette available in this font. Please submit math equations as editable text and not as images. Tables and figure legends should be placed in a separate section at the end of the manuscript (not placed within the text). Figure files should be uploaded as separate files (not embedded in the manuscript).

Use of word-processing software:

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in

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Second subheadings begin the first line of a paragraph. They are indented, boldface, italic, and followed by a period. The first letter of each important word should be capitalized. The text follows immediately after the final period of the subheading.

Title Page

The title page shall begin with a running head (short title) of not more than 45 characters. The running head is centered, is in all capital letters, and shall appear on the top of the title page. No abbreviations should be used.

The title of the paper must be in boldface; the first letter of the article title and proper names are capitalized, and the remainder of the title is lowercase. The title must not have abbreviations.

Under the title, names of authors should be typed (first name or initial, middle initial, last name). Affiliations will be footnoted using the following symbols: *, †, ‡, §, #, ||, and be placed below the author names. Do not give authors' titles, positions, or degrees. Numbered footnotes may be used to provide supplementary information, such as present address, acknowledgment of grants, and experiment station or journal series number. The corresponding author should be indicated with a numbered footnote (e.g., Corresponding author: name@university.edu). Note: *Poultry Science* allows a single corresponding author; co-corresponding authors are not permitted.

In some instances, the first two authors of a manuscript may be designated as equal contributors. However, the corresponding author should be prepared to justify such designation, if asked by the editorial staff. More than two equal contributors is not permitted under any circumstance.

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author being added or removed. Once a paper reaches the proof stage, no changes to the author list are permitted.

Abbreviations

Author-derived abbreviations should be defined at first use in the abstract and again in the body of the manuscript. The abbreviation will be shown in bold type at first use in the body of the manuscript. Refer to the Miscellaneous Usage Notes for more information on abbreviations.

Abstract

The Abstract disseminates scientific information through abstracting journals and through convenience for the readers. The Abstract, consisting of not more than 325 words, appears at the beginning of the manuscript with the word ABSTRACT without a following period. It must summarize the major objectives, methods, results, conclusions, and practical applications of the research. The Abstract must consist of complete sentences and use of abbreviations should be limited. References to other work and footnotes are not permitted. The Abstract and Key Words must be on a separate sheet of paper.

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The Abstract shall be followed by a maximum of five key words or phrases to be used for subject indexing. These should include important words from the title and the running head and should be singular, not plural, terms (e.g., broiler, not broilers). Key words should be formatted as follows: Key words: . . .

Article Structure

Introduction

The Introduction, while brief, should provide the reader with information necessary for understanding research presented in the paper. Previous work on the topic should be summarized, and the objectives of the current research must be clearly stated.

Materials and methods

All sources of products, equipment, and chemicals used in the experiments must be specified parenthetically at first mention in text, tables, and figures [i.e., (model 123, ABC Corp., Provo, UT)]. Model and catalog numbers should be included. Information shall include the full corporate name (including division, branch, or other subordinate part of the corporation, if applicable), city, and state (country if outside the United States), or Web address. Street addresses need not be given unless the reader would not be able to determine the full address for mailing purposes easily by consulting standard references.

Age, sex, breed, and strain or genetic stock of animals used in the experiments shall be specified. Animal care guidelines should be referenced if appropriate.

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When describing the composition of diets and vitamin premixes, the concentration of vitamins A and E should be expressed as IU/kg on the basis of the following equivalents:

Vitamin A

1 IU = 0.3 µg of all-trans retinol 1 IU = 0.344 µg of retinyl acetate

1 IU = 0.552 µg of retinyl palmitate

1 IU = 0.60 µg of ß-carotene

Vitamin E

1 IU = 1 mg of dl-α-tocopheryl acetate 1 IU = 0.91 mg of dl-α-tocopherol

1 IU = 0.67 mg of d-α-tocopherol

In the instance of vitamin D3, cholecalciferol is the acceptable term on the basis that 1 IU of vitamin D3 = 0.025 µg of cholecalciferol.

The sources of vitamins A and E must be specified in parentheses immediately following the stated concentrations.

• **Statistical analysis:** Biology should be emphasized, but the use of incorrect or inadequate statistical methods to analyze and interpret biological data is not acceptable. Consultation with a statistician is recommended. Statistical methods commonly used in the animal sciences need not be described in detail, but adequate references should be provided. The statistical model, classes, blocks, and experimental unit must be designated. Any restrictions used in estimating parameters should be defined. Reference to a statistical package without reporting the sources of variation (classes) and other salient features of the analysis, such as covariance or orthogonal contrasts, is not sufficient. A statement of the results of statistical analysis should justify the interpretations and conclusions.

When possible, results of similar experiments should be pooled statistically. Do not report a number of similar experiments separately.

The experimental unit is the smallest unit to which an individual treatment is imposed. For group-fed animals, the group of animals in the pen is the experimental unit; therefore, groups must be replicated. Repeated chemical analyses of the same sample usually do not constitute independent experimental units. Measurements on the same experimental unit over time also are not independent and must not be considered as independent experimental units. For analysis of time effects, use time-sequence analysis.

• Usual assumptions are that errors in the statistical models are normally and independently distributed with constant variance. Most standard methods are robust to deviations from these assumptions, but occasionally data transformations or other techniques are helpful. For example, it is recommended that percentage data between 0 and 20 and between 80 and 100 be subjected to arc sin transformation prior to analysis. Most statistical procedures are based on the assumption that experimental units have been assigned to treatments at random. If animals are stratified by ancestry or weight or if some other initial

measurement should be accounted for, they should include a blocking factor, or the initial measurement should be included as a covariate.

• A parameter [mean (μ), variance (ó2)], which defines or describes a population, is estimated by a statistic (x, s2). The term parameter is not appropriate to describe a variable, observation, trait, characteristic, or measurement taken in an experiment.

• Standard designs are adequately described by name and size (e.g., "a randomized complete block design with 6 treatments in 5 blocks"). For a factorial set of treatments, an adequate description might be as follows: "Total sulfur amino acids at 0.70 or 0.80% of the diet and Lys at 1.10, 1.20, or 1.30% of the diet were used in a 2 x 3 factorial arrangement in 5 randomized complete blocks consisting of initial BW." Note that a factorial arrangement is not a design; the term "design" refers to the method of grouping experimental units into homogeneous groups or blocks (i.e., the way in which the randomization is restricted).

• Standard deviation refers to the variability in a sample or a population. The standard error (calculated from error variance) is the estimated sampling error of a statistic such as the sample mean. When a standard deviation or standard error is given, the number of degrees of freedom on which it rests should be specified. When any statistical value (as mean or difference of 2 means) is mentioned, its standard error or confidence limit should be given. The fact that differences are not "statistically significant" is no reason for omitting standard errors. They are of value when results from several experiments are combined in the future. They also are useful to the reader as measures of efficiency of experimental techniques. A value attached by "±" to a number implies that the second value is its standard error (not its standard deviation). Adequate reporting may require only 1) the number of observations, 2) arithmetic treatment means, and 3) an estimate of experimental error. The pooled standard error of the mean is the preferred estimate of experimental error. Standard errors need not be presented separately for each mean unless the means are based on different numbers of observations or the heterogeneity of the error variance is to be emphasized. Presenting individual standard errors clutters the presentation and can mislead readers.

• For more complex experiments, tables of subclass means and tables of analyses of variance or covariance may be included. When the analysis of variance contains several error terms, such as in split-plot and repeated measures designs, the text should indicate clearly which mean square was used for the denominator of each F statistic. Unbalanced factorial data can present special problems. Accordingly, it is well to state how the computing was done and how the parameters were estimated. Approximations should be accompanied by cautions concerning possible biases.

• Contrasts (preferably orthogonal) are used to answer specific questions for which the experiment was designed; they should form the basis for comparing treatment means. Nonorthogonal contrasts may be evaluated by Bonferroni t statistics. The exact contrasts tested should be described for the reader. Multiple-range tests are not appropriate when treatments are orthogonally arranged. Fixed-range, pairwise, multiple-comparison tests should be used only to compare means of treatments that are unstructured or not related. Least squares means are the correct means to use for all data, but arithmetic means are identical to least squares means unless the design is unbalanced or contains missing values or an adjustment is being made for a covariate. In factorial treatment arrangements, means for main effects should be presented when important interactions are not present. However, means for individual treatment combinations also should be provided in table or text so that future researchers may combine data from several experiments to detect important interactions. An interaction may not be detected in a given experiment because of a limitation in the number of observations.

• The terms significant and highly significant traditionally have been reserved for $P < 0.05$ and $P < 0.01$, respectively; however, reporting the P-value is preferred to the use of these terms. For example, use ". . . there was a difference ($P < 0.05$) between control and treated samples" rather than ". . . there was a significant ($P < 0.05$) difference between control and treated samples." When available, the observed significance level (e.g., $P = 0.027$) should be presented rather than merely $P < 0.05$ or $P < 0.01$, thereby allowing the reader to decide what to reject. Other probability (α) levels may be discussed if properly qualified so that the reader is not misled. Do not report P-values to more than 3 places after the

decimal. Regardless of the probability level used, failure to reject a hypothesis should be based on the relative con- sequences of type I and II errors. A "nonsignificant" relationship should not be interpreted to suggest the absence of a relationship. An inadequate number of experimental units or insufficient control of variation limits the power to detect relationships. Avoid the ambiguous use of $P > 0.05$ to declare nonsignificance, such as indicating that a difference is not significant at $P > 0.05$ and subsequently declaring another difference significant (or a tendency) at $P < 0.09$. In addition, readers may incorrectly interpret the use of $P > 0.05$ as the probability of a β error, not an α error.

• Present only meaningful digits. A practical rule is to round values so that the change caused by rounding is less than one-tenth of the standard error. Such rounding increases the variance of the reported value by less than 1%, so that less than 1% of the relevant information contained in the data is sacrificed. Significant digits in data reported should be restricted to 3 beyond the decimal point, unless warranted by the use of specific methods.

Results and discussion

Results and Discussion sections may be combined, or they may appear in separate sections. If separate, the Results section shall contain only the results and summary of the author's experiments; there should be no literature comparisons. Those comparisons should appear in the Discussion section. Manuscripts reporting sequence data must have GenBank accession numbers prior to submitting. One of the hallmarks for experimental evidence is repeatability. Care should be taken to ensure that experiments are adequately replicated. The results of experiments must be replicated, either by replicating treatments within experiments or by repeating experiments.

Acknowledgements

An Acknowledgments section, if desired, shall follow the Discussion section. Acknowledgments of individuals should include affiliations but not titles, such as Dr., Mr., or Ms. Affiliations shall include institution, city, and state.

References

Citations in text

In the body of the manuscript, refer to authors as follows: Smith and Jones (1992) or Smith and Jones (1990, 1992). If the sentence structure requires that the authors' names be included in parentheses, the proper format is (Smith and Jones, 1982; Jones, 1988a,b; Jones et al., 1993). Where there are more than two authors of one article, the first author's name is followed by the abbreviation et al. More than one article listed in the same sentence of text must be in chronological order first, and alphabetical order for two publications in the same year. Work that has not been accepted for publication shall be listed in the text as: "J. E. Jones (institution, city, and state, personal communication)." The author's own un-published work should be listed in the text as "(J. Smith, unpublished data)." Personal communications and un- published data must not be included in the References section.

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N.B. - The online version of Poultry Science uses a reference format that differs from that prescribed by the journal. The Guide for Authors is the sole source for the reference format. Any papers that do not follow this format risk rejection.

Article:

Bagley, L. G., and V. L. Christensen. 1991. Hatchability and physiology of turkey embryos incubated at sea level with increased eggshell permeability. Poult. Sci. 70:1412-1418.

Bagley, L. G., V. L. Christensen, and R. P. Gildersleeve. 1990. Hematological indices of turkey embryos incubated at high altitude as affected by oxygen and shell permeability. Poult. Sci. 69:2035- 2039.

Witter, R. L., and I. M. Gimeno. 2006. Susceptibility of adult chickens, with and without prior vaccination, to challenge with Marek's disease virus. Avian Dis. 50:354-365. doi:10.1637/7498-010306R.1

Book:

Metcalfe, J., M. K. Stock, and R. L. Ingermann. 1984. The effects of oxygen on growth and development of the chick embryo. Pages 205- 219 in Respiration and Metabolism of Embryonic Vertebrates. R. S. Seymour, ed. Dr. W. Junk, Dordrecht, the Netherlands.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Federal Register:

Department of Agriculture, Plant and Animal Health Inspection Service. 2004. Blood and tissue collection at slaughtering and rendering establishments, final rule. 9CFR part 71. Fed. Regis. 69:10137-10151.

Other:

Choct, M., and R. J. Hughes. 1996. Long-chain hydrocarbons as a marker for digestibility studies in poultry. Proc. Aust. Poult. Sci. Symp. 8:186. (Abstr.)

Dyro, F. M. 2005. Arsenic. WebMD. Accessed Feb. 2006. http:// www.emedicine.com/neuro/topic20.htm.

El Halawani, M. E., and I. Rosenboim. 2004. Method to enhance reproductive performance in poultry. Univ. Minnesota, as- signee. US Pat. No. 6,766,767.

Hruby, M., J. C. Remus, and E. E. M. Pierson. 2004. Nutritional strategies to meet the challenge of feeding poultry without antibiotic growth promotants. Proc. 2nd Mid-Atlantic Nutr. Conf., Timonium, MD. Univ. Maryland, College Park.

Luzuriaga, D. A. 1999. Application of computer vision and electronic nose technologies for quality assessment of color and odor of shrimp and salmon. PhD Diss. Univ. Florida, Gainesville.

Peak, S. D., and J. Brake. 2000. The influence of feeding program on broiler breeder male mortality. Poult. Sci. 79(Suppl. 1):2. (Abstr.)

Tables

Tables must be created using the MS Word table feature and inserted in the manuscript after the references section. When possible, tables should be organized to fit across the page without running broadside. Be aware of the dimensions of the printed page when planning tables (use of more than 15 columns will create layout problems). Place the table number and title on the same line above the table. The table title does not require a period. Do not use vertical lines and use few horizontal lines. Use of bold and italic typefaces in the table should be done sparingly; you must define such use in a footnote. Each table must be on a separate page. To facilitate placement of all tables into the manuscript file (just after the references) authors should use "section breaks" rather than "page breaks" at the end of the manuscript (before the tables) and between tables.

Units of measure for each variable must be indicated. Papers with several tables must use consistent format. All columns must have appropriate headings. Abbreviations not found on the inside front cover of the journal must be defined in each table and must match those used in the text. Footnotes to tables should be marked by superscript numbers. Each footnote should begin a new line. Superscript letters shall be used for the separation of means in the body of the table and explanatory footnotes must be provided [i.e., "Means within a row lacking a common superscript differ ($P < 0.05$)."]; other significant P-values may be specified. Comparison of means within rows and columns should be indicated by different series of superscripts (e.g., a,b,… in rows; x-z … in columns) The first alphabetical letter in the series (e.g., a or A) shall be used to indicate the largest

mean. Lowercase super- scripts indicate P ≤ 0.05. Uppercase letters indicate P ≤ 0.01 or less.

Probability values may be indicated as follows: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and †P ≤ 0.10. Consult a recent issue of *Poultry Science* for examples of tables.

Generally, results should be presented to the significant figure of the instrument used to collect the data. For example, results should not be presented to 5 digits when the instrument used only reads to 2 digits.

Miscellaneous Usage Notes

Abbreviations

• Abbreviations shall not be used in the title, key words, or to begin sentences, except when they are widely known throughout science (e.g., DNA, RNA) or are terms better known by abbreviation (e.g., IgG, CD). A helpful criterion for use of abbreviation is whether it has been accepted into thesauri and indexes widely used for searching major bibliographic databases in the scientific field. Abbreviations may be used in heads within the paper, if they have been first defined within the text. The inside back cover of every issue of the journal lists abbreviations that can be used without definition. The list is subject to revision at any time, so authors should always consult the most recent issue of the journal for relevant information. Abbreviations are allowed when they help the flow of the manuscript; however, excessive use of abbreviations can confuse the reader. The suitability of abbreviations will be evaluated by the reviewers and editors during the review process and by the technical editor during editing. As a rule, author-derived abbreviations should be in all capital letters. Terms used less than three times must be spelled out in full rather than abbreviated. All terms are to be spelled out in full with the abbreviation following in bold type in parentheses the first time they are mentioned in the main body of the text. Abbreviations shall be used consistently thereafter, rather than the full term.

• The abstract, text, each table, and each figure must be understood independently of each other. Therefore, abbreviations shall be defined within each of these units of the manuscript.

• Plural abbreviations do not require "s." Chemical symbols and three-letter abbreviations for amino acids do not need definition. Units of measure, except those in the standard *Poultry Science* abbreviation list, should be abbreviated as listed in the CRC Handbook for Chemistry and Physics (CRC Press, 2000 Corporate Blvd., Boca Raton, FL, 33431) and do not need to be defined.

• The following abbreviations may be used without definition in *Poultry Science*:

A adenine

ADG average daily gain

ADFI average daily feed

AME apparent metabolizable energy

AMEn nitrogen-corrected apparent metabolizable energy

ANOVA analysis of variance

B cell bursal-derived, bursal-equivalent derived cell bp base pairs

BSA bovine serum albumin

BW body weight

C cytosine

cDNA complementary DNA

cfu colony-forming units

CI confidence interval

CP crude protein

cpm counts per minute

CV coefficient of variation

d day

df degrees of freedom

DM dry matter

DNA deoxyribonucleic acid

EDTA ethylenediaminetetraacetate

ELISA enzyme-linked immunosorbent antibody assay

EST expressed sequence tag

g gram

g gravity

G guanine

GAT glutamic acid-alanine-tyrosine

GLM general linear model

h hour

HEPES N-2-hydroxyethyl piperazine-N'-ethane-sulfonic acid

HPLC high-performance (high-pressure) liquid chromatography

i.m. intramuscular

i.p. intraperitoneal

i.v. intravenous

ICU international chick units

Ig immunoglobulin

IL interleukin

IU international units

kb kilobase pairs

kDa kilodalton

L liter*

L:D hours light:hours darkness in a photoperiod (e.g., 23L:1D)

m meter

µ micro M molar

MAS marker-assisted selection

ME metabolizable energy

MEn nitrogen-corrected metabolizable energy

MHC major histocompatibility complex

mRNA messenger ribonucleic acid

min minute

mo month

MS mean square

n number of observations

N normal

NAD nicotinamide adenine dinucleotide

NADH reduced nicotinamide adenine dinucleotide

NRC National Research Council

NS not significant

PAGE polyacrylamide gel electrophoresis

PBS phosphate-buffered saline

PCR polymerase chain reaction

pfu plaque-forming units

ppm parts per million

QTL quantitative trait loci

r correlation coefficient

r2 coefficient of determination, simple

R2 coefficient of determination, multiple

RH relative humidity

RIA radioimmunoassay

RNA ribonucleic acid

rpm revolutions per minute

s second

s.c. subcutaneous

SD standard deviation

SDS sodium dodecyl sulphate

SE standard error

SEM standard error of the mean

SRBC sheep red blood cells

SNP single nucleotide polymorphism

T thymine

TBA thiobarbituric acid

T cell thymic-derived cell

TME true metabolizable energy

TMEn nitrogen-corrected true metabolizable energy

Tris tris(hydroxymethyl)aminomethane

TSAA total sulfur amino acids

U uridine

USDA United States Department of Agriculture

UV ultraviolet

vol/vol volume to volume

vs. versus

wt/vol weight to volume

wt/wt weight to weight

wk week

yr year

*Also capitalized with any combination, e.g., mL.

International words and phrases

Non-English words in common usage (defined in recent editions of standard dictionaries) will not appear in italics (e.g., in vitro, in vivo, in situ, a priori). However, genus and species of plants, animals, or bacteria and viruses should be italicized. Authors must indicate accent marks and other diacriticals on international names and institutions. German nouns shall begin with capital letters.

Capitalization

Breed and variety names are to be capitalized (e.g., Single Comb White Leghorn).

Number style

Numbers less than 1 shall be written with preceding zeros (e.g., 0.75). All numbers shall be written as digits. Measures must be in the metric system; however, US equivalents may be given in parentheses. *Poultry Science* requires that measures of energy be given in calories rather than joules, but the equivalent in joules may be shown in parentheses or in a footnote to tables. Units of measure not preceded by numbers must be written out rather than abbreviated (e.g., lysine content was measured in milligrams per kilogram of diet) unless used parenthetically. Measures of variation must be defined in the Abstract and in the body of the paper at first use. Units of measure for feed conversion or feed efficiency shall be provided (i.e., g:g).

Nucleotide sequences

Nucleotide sequence data must relate to poultry or poultry pathogens and must complement biological data published in the same or a companion paper. If sequences are excessively long, it is suggested that the most relevant sections

of the data be published in *Poultry Science* and the remaining sequences be submitted to one of the sequence databases. Acceptance for publication is contingent on the submission of sequence data to one of the databases. The following statement should appear as a footnote to the title on the title page of the manuscript. "The nucleotide sequence data reported in this paper have been submitted to Embank Submission (Mail Stop K710, Los Alamos National Laboratories, Los Alamos, NM 87545) nucleotide sequence database and have been assigned the accession number XNNNNN." Publication of the description of molecular clones is assumed by the editors to place them in the public sector. Therefore, they shall be made available to other scientists for research purposes.

Nucleotide sequences must be submitted as camera- ready figures no larger than 21.6 x 27.9 cm in standard (portrait) orientation. Abbreviations should follow *Poultry Science* guidelines.

Gene and protein nomenclature

Authors are required to use only approved gene and protein names and symbols. For poultry, full gene names should not be italicized. Gene symbols should be in uppercase letters and should be in italics. A protein symbol should be in the same format as its gene except the protein symbol should not be in italics.

General usage

• Note that "and/or" is not permitted; choose the more appropriate meaning or use "x or y or both."

• Use the slant line only when it means "per" with numbered units of measure or "divided by" in equations. Use only one slant line in a given expression (e.g., g/d per chick). The slant line may not be used to indicate ratios or mixtures.

• Use "to" instead of a hyphen to indicate a range. Insert spaces around all signs (except slant lines) of operation (=, -, +, x, >, or <, etc.) when these signs occur between two items.

• Items in a series should be separated by commas (e.g., a, b, and c).

• Restrict the use of "while" and "since" to meanings related to time.

• Appropriate substitutes include "and," "but," or "whereas" for "while" and "because" or "although" for "since."

• Leading (initial) zeros should be used with numbers less than 1 (e.g., 0.01).

• Commas should be used in numbers greater than 999.

•Registered (®) and trademark (©) symbols should not be used, unless as part of an article title in the References section. Trademarked product names should be capitalized.

Figures/Illustrations

General points

• Submit each illustration as a separate file.

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.

• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Size the illustrations close to the desired dimensions of the published version.

A detailed guide on electronic artwork is available.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of

the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.

TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.

TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

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'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Supplementary Data

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Ensure that the supplementary material is referred to in the main manuscript at an appropriate point in the text. Supplementary material will be available online only and will not be copyedited, so ensure that it is clearly and succinctly presented, and that the style conforms to the rest of the paper. Also ensure that the presentation will work on any Internet browser. It is not recommended for the files to be more than 2 MB each, although exceptions can be made at the editorial office's discretion.

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Corresponding authors will receive an e-mail with a link to their proof. Please annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes

to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Articles In Press

For *Poultry Science*, manuscripts arrive at Elsevier and go through the production process until the final versions are ready to publish. These are then published on the journal's articles in press page. They will remain on the page up until the issue that they are assigned to is published.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication.

Reporting sex- and gender-based analyses

Reporting guidance

For research involving or pertaining to humans, animals or eukaryotic cells, investigators should integrate sex and gender-based analyses (SGBA) into their research design according to funder/sponsor requirements and best practices within a field. Authors should address the sex and/or gender dimensions of their research in their article. In cases where they cannot, they should discuss this as a limitation to their research's generalizability. Importantly, authors should explicitly state what definitions of sex and/or gender they are applying to enhance the precision, rigor and reproducibility of their research and to avoid ambiguity or conflation of terms and the constructs to which they refer (see Definitions section below). Authors can refer to the Sex and Gender Equity in Research (SAGER) guidelines and the SAGER guidelines checklist. These offer systematic approaches to the use and editorial review of sex and gender information in study

design, data analysis, outcome reporting and research interpretation - however, please note there is no single, universally agreed-upon set of guidelines for defining sex and gender.

Definitions

Sex generally refers to a set of biological attributes that are associated with physical and physiological features (e.g., chromosomal genotype, hormonal levels, internal and external anatomy). A binary sex categorization (male/female) is usually designated at birth ("sex assigned at birth"), most often based solely on the visible external anatomy of a newborn. Gender generally refers to socially constructed roles, behaviors, and identities of women, men and gender-diverse people that occur in a historical and cultural context and may vary across societies and over time. Gender influences how people view themselves and each other, how they behave and interact and how power is distributed in society. Sex and gender are often incorrectly portrayed as binary (female/male or woman/man) and unchanging whereas these constructs actually exist along a spectrum and include additional sex categorizations and gender identities such as people who are intersex/have differences of sex development (DSD) or identify as non-binary. Moreover, the terms "sex" and "gender" can be ambiguous--thus it is important for authors to define the manner in which they are used. In addition to this definition guidance and the SAGER guidelines, the resources on this page offer further insight around sex and gender in research studies.