

Implications of elevated threonine plasma concentrations in the development of reduced-crude protein diets for broiler chickens

Shemil P. Macelline^{A,B}, Peter V. Chrystal^{A,C}, Sonia Yun Liu^{A,B} and Peter H. Selle^{ID A,D,E}

^APoultry Research Foundation within The University of Sydney, Camden Campus, 425 Werombi Road, NSW 2570, Australia.

^BSchool of Life and Environmental Sciences, Faculty of Science, The University of Sydney, NSW 2006, Australia.

^CBaiaida Poultry Pty Limited, Pendle Hill, NSW 2145, Australia.

^DSydney School of Veterinary Science, The University of Sydney, Sydney, NSW 2006, Australia.

^ECorresponding author. Email: peter.selle@sydney.edu.au

Abstract. There is a real quest to develop reduced-crude protein diets to facilitate sustainable chicken-meat production. However, pronounced elevations in threonine plasma concentrations in systemic plasma have consistently been observed pursuant to crude protein reductions in diets for broiler chickens. The aim of the present Perspective was to consider the genesis and consequences of these elevated threonine concentrations. A series of five reduced-crude protein feeding studies with maize-based diets completed on the Camden Campus of Sydney University was the basis of the present Perspective. Collectively, an average reduction in dietary crude protein from 212 to 167 g/kg generated a mean increase of 64.8% (867 versus 526 $\mu\text{mol/L}$) in threonine plasma concentrations. This was attributed to the downregulation of hepatic threonine dehydrogenase activity, which catalyses threonine to acetyl-CoA and glycine and a mechanism for this inhibition is proposed. Tangible reductions in dietary crude protein usually impair feed conversion efficiency and increase fat deposition. Threonine plasma concentrations are elevated by these reductions and the likelihood is that threonine concentrations may be an indicative biomarker of the precision with which efficient reduced-CP broiler diets are formulated and, if so, would facilitate their successful development.

Keywords: amino acids, broiler chickens, glucose, protein, starch, threonine.

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Introduction

There is considerable interest in the development of reduced-crude protein (CP) diets for broiler chickens for which there is a compelling justification as outlined by Greenhalgh *et al.* (2020a). The successful development of reduced-CP diets will attenuate emissions of nitrogen and ammonia, which is environmentally advantageous. Reduced-CP diets will enhance litter quality and reduce the incidence of foot-pad lesions. Poor litter quality is a complex problem and is detrimental to bird welfare, which is an issue of increasing importance (Dunlop *et al.* 2016). Furthermore, reduced-CP diets have the potential to reduce the dependence of chicken-meat industry on soybean meal (Selle *et al.* 2020). This would be a real advantage in countries that rely on importations of soybean meal. Nevertheless, the successful development of reduced-CP diets is fraught with challenges, as considered by Chrystal *et al.* (2020a), because dietary CP reductions usually compromise feed conversion ratios (FCR), with associated increases in fat deposition.

Threonine is the third limiting amino acid in diets for broiler chickens after methionine and lysine (Kidd and Kerr 1996) and constitutes 36.3 g/kg of whole-body protein in broiler chickens (Wu 2014). Additionally, threonine is the dominant amino acid in avian mucin (Fang *et al.* 1993). The importance of threonine was demonstrated by free threonine concentrations in portal plasma taken from the anterior mesenteric vein (Selle *et al.* 2016). Increasing portal threonine plasma concentrations were linearly associated with both enhanced weight gain ($r = 0.915$, $P < 0.0001$) and feed conversion efficiency ($r = -0.773$, $P < 0.01$) in broilers from 7 to 28 days post-hatch in this study.

Free amino acid concentrations in systemic plasma are indicative of protein or amino acid utilisation in broiler chickens (Dean and Scott 1966; Fernández-Fígares *et al.* 1997). However, an intriguing outcome was reported by Fancher and Jensen (1989) in which female broilers were offered maize-based diets containing either 183 or 159 g/kg CP. Three 159 g/kg CP diets containing 1.3 g/kg non-bound

threonine and different inclusions of other non-bound (synthetic, crystalline) amino acids, were compared with one 183 g/kg CP diet. The transition from 183 to 159 g/kg CP diets generated an average increase in systemic threonine plasma concentration of 124% (1959 versus 876 $\mu\text{mol/L}$), whereas plasma concentrations of the balance of amino acids assessed essentially remained static. Thus, this outcome raises the obvious question as to the genesis of these elevated threonine plasma concentrations pursuant to reductions in dietary CP. Moreover, this conundrum became increasingly more relevant when similar elevations in threonine plasma concentrations were repeatedly observed in broiler chickens offered maize-based diets on the Camden Campus of Sydney University. Therefore, the purpose of the present paper was to review these outcomes and to consider their genesis and implications of elevated threonine plasma concentrations in the context of the development of reduced-CP diets for sustainable chicken-meat production.

Impact of dietary CP reductions on threonine plasma concentrations

In a series of five feeding studies, off-sex, male Ross 308 chickens were offered maize-based diets from 7 to 35 days post-hatch, with dietary CP concentrations ranging from 222 to 156 g/kg (Moss *et al.* 2018; Chrystal *et al.* 2020b; 2020c, 2020d, 2021). The relevant data are presented in Table 1 where the dietary mean molar concentration of threonine was 68.3 mmolar, with a standard deviation of ± 4.87 . In contrast, free threonine plasma concentrations ranged from 428 to 1093 $\mu\text{mol/L}$ around a mean value of 664 $\mu\text{mol/L} \pm 189.9$ in systemic plasma. Instructively, there is a quadratic relationship ($r = 0.797$, $P < 0.001$) between analysed dietary CP concentrations and free threonine plasma concentrations, as shown in Fig. 1. The regression equation predicts that

elevations of threonine plasma concentrations will be observed once dietary CP concentrations are reduced below 206 g/kg. When the highest- and lowest-CP diets in each experiment were compared, analysed CP concentrations decreased by an average of 42 g/kg from 212 to 170 g/kg and, in response, free threonine plasma concentrations increased by an average of 64.8% (867 versus 526 $\mu\text{mol/L}$). Across the individual experiments, elevations in threonine plasma concentrations ranged from 39.6% (863 versus 396 $\mu\text{mol/L}$) in Chrystal *et al.* (2021) to 116.4% (1027 versus 619 $\mu\text{mol/L}$) in Chrystal *et al.* (2020c). Clearly, the elevations in threonine plasma concentrations are variable; however, they are related to both FCR and relative fat-pad weights. From the same lowest to highest CP comparison, FCR were compromised by 4.29% (1.545 versus 1.481) and fat-pad weights were 77.6% heavier in the lowest CP diet (13.28 versus 7.48 g/kg). Compromised efficiency of feed conversion and heavier relative fat-pad weights are typical responses to reductions in dietary CP, so it is noteworthy that threonine plasma concentrations are quadratically related to heavier relative fat-pad weights ($r = 0.680$, $P = 0.024$) and compromised FCR ($r = 0.569$, $P = 0.065$), when the tabulated data are considered in their entirety. This is not to suggest that elevated threonine plasma concentrations are generating these critical negative responses, but they may be indicative of compromised performance when birds are offered reduced-CP diets.

The molar proportions of free threonine relative to total amino acid concentrations in systemic plasma are illustrated in Fig. 2. In Studies 1–4, molar proportions of plasma threonine increased in an essentially linear fashion in response to declining dietary CP from an average of 8.48% to 14.16%, which is an increase of 67.0%, when the highest- and lowest-CP diets in each experiment were considered. In Study 5

Table 1. A tabulated summary of relevant data from five reduced-CP feeding studies with maize-based diets

Study 1, Moss *et al.* (2018); Study 2, Chrystal *et al.* (2020b); Study 3, Chrystal *et al.* (2020c); Study 4, Chrystal *et al.* (2020d); Study 5, Chrystal *et al.* (2021)

Study	Specified dietary CP (g/kg)	Analysed dietary threonine (g/kg)	Plasma threonine ($\mu\text{mol/L}$)	Plasma glycine ($\mu\text{mol/L}$)	Analysed dietary starch (g/kg)	Analysed dietary CP (g/kg)	Starch:protein ratio	FCR (g/g)	Fat-pad weight (g/kg)
Study 1	213	72	428	797	269	219	1.23	1.379	n.a.
	178	70	689	805	431	190	2.27	1.452	n.a.
Study 2	210	75	619	769	312	216	1.45	1.569	8.64
	195	72	514	646	327	195	1.64	1.559	12.01
	180	70	799	668	362	188	1.95	1.538	12.57
	165	66	1027	535	395	170	2.30	1.608	14.62
Study 3	200	70	505	675	303	204	1.48	1.495	7.26
	188	66	631	581	323	183	1.76	1.500	8.49
	172	66	715	484	356	174	2.05	1.522	10.13
	156	65	1093	415	399	157	2.54	1.629	12.40
Study 4	208	63	461	669	356	197	1.81	1.510	7.62
	193	63	500	618	373	189	1.97	1.523	9.57
	179	62	512	549	406	180	2.26	1.528	9.90
	165	60	664	606	409	163	2.51	1.562	13.31
Study 5	222	76	618	614	326	224	1.46	1.453	6.4
	193	70	648	701	370	192	1.93	1.415	11.1
	165	75	863	753	448	169	2.65	1.473	12.8

(Chrystal *et al.* 2021), both maize and wheat-based diets were investigated where the 66.1% (19.91 versus 11.99%) increase in threonine molar proportions with wheat was more pronounced than the 24.1% (14.18 versus 11.43%) increase with maize-based diets. This difference between wheat and maize is discussed later.

A rationale for elevated threonine plasma concentrations

Threonine is an essential amino acid (Malinovsky 2018); therefore, the elevations in plasma threonine concentrations illustrated in Fig. 1 cannot stem from the biosynthesis of threonine in poultry. Alternatively, the elevations could be the result of decreased threonine catabolism where potentially three enzymes have the capacity to catabolise threonine in the liver. These include threonine dehydratase, which

degrades threonine to α -ketobutyrate; threonine aldolase, which degrades threonine to acetaldehyde and glycine; and threonine dehydrogenase (TDH), which degrades threonine to acetyl-CoA and glycine (Davis and Austic 1982). Threonine is principally degraded by TDH in poultry; instructively, Akagi *et al.* (2004) found hepatic TDH activity (88%) to be dominant in avian species (Japanese quail). In contrast, threonine dehydratase (93%) was the dominant enzyme in rats. Akagi *et al.* (2004) classified threonine as a ketogenic amino acid in avian species because TDH metabolises to acetyl-CoA, whereas threonine is usually regarded as a glucogenic amino acid in mammals (D'Andrea 2000). Thus, it is plausible that elevated free threonine plasma concentrations in broiler chickens are essentially generated by the downregulation of hepatic TDH activity.

Several investigations into TDH activity in poultry have been completed (Davis and Austic 1997; Yuan *et al.* 2000; Yuan and Austic 2001; Lee *et al.* 2011, 2014, 2016) without reaching finite conclusions. The activity of TDH in hepatic mitochondria was specifically examined in Davis and Austic (1997) and it was suggested that hepatic TDH activity is influenced more so by concentrations of dietary protein or other amino acids than by threonine *per se*. Yuan and Austic (2001) reported that lowering dietary CP from 320 to 230 g/kg reduced total TDH activity in hepatic mitochondria of chickens by 48.3% (18.3 versus 35.4 units). This would be expected to elevate free threonine plasma concentrations, but a dietary CP of 320 g/kg is very much higher than standard. However, Lee *et al.* (2014) found that TDH activity in chicks was stimulated by additional dietary threonine and protein and, presumably, lowering dietary concentrations of threonine and/or protein would have the reverse effect. The mean analysed dietary threonine concentration was 8.16 g/kg and ranged from 7.4 to 9.1 g/kg in the five nominated experiments; therefore, there were no gross variations in dietary threonine concentrations in association with dietary CP reductions.

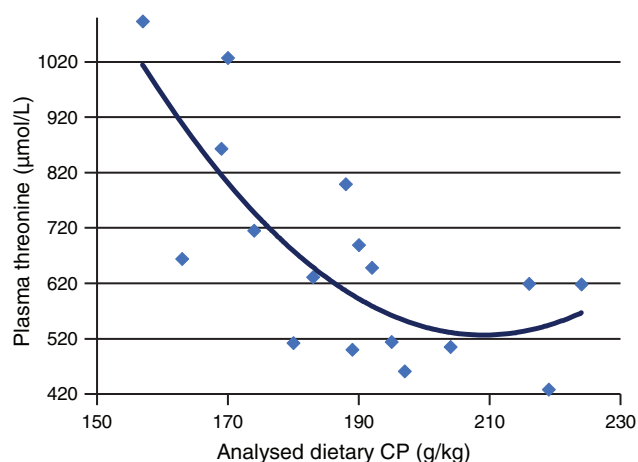


Fig. 1. Quadratic relationship ($r = 0.797$; $P < 0.001$) between analysed dietary CP concentrations and free threonine concentrations in systemic plasma where $y_{(\text{thr } \mu\text{mol/L})} = 9103 - 83.152 \times \text{CP}_{(\text{g/kg})} + 0.202 \times \text{CP}_{(\text{g/kg})}^2$.

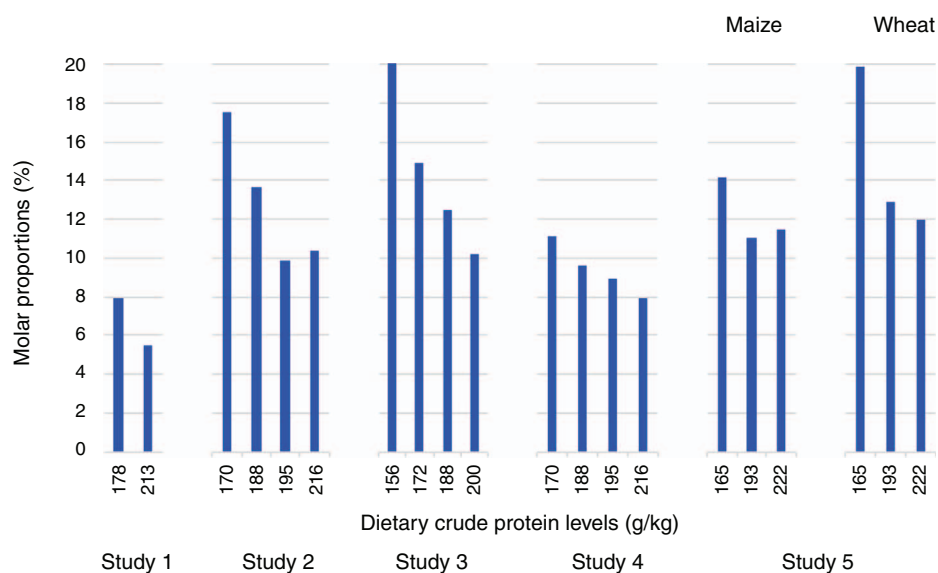


Fig. 2. Molar proportions of free threonine plasma concentrations in broiler chickens offered a range of dietary crude protein concentrations in five experiments.

However, the above findings suggest that reductions in dietary protein may depress TDH activity.

Of relevance is that Guerranti *et al.* (2001) investigated the inhibition of hepatic TDH activity in rats in a study where the focus was on fatty acids. These researchers concluded that acetyl-CoA and its derivatives depressed TDH activity by selective feedback inhibition as acetyl-CoA is a major end product of threonine catabolism. However, the catabolism of ketogenic amino acids, and specifically threonine by TDH, generates acetyl-CoA, which is a central metabolic intermediate capable of influencing the activity of numerous enzymes (Pietrocola *et al.* 2015). Pivotaly, glucose may be metabolised to acetyl-CoA (Shi and Tu 2015), and Kaempfer *et al.* (1991) investigated the fraction of hepatic acetyl-CoA that is derived from glucose in rats. These researchers found that the quantity of acetyl-CoA derived from glucose fluctuates with the availability of carbohydrate. Typically, dietary starch:protein ratios, starch, and potentially glucose, concentrations are increased in reduced-CP diets, while fat concentrations are decreased. From the data in Table 1, analysed dietary starch concentrations increase by 33.0% (416 versus 313 g/kg) and analysed dietary starch:protein ratios increase from 1.49 to 2.45 when the lowest- to highest-CP diets are compared. Therefore, high starch/glucose concentrations in birds offered reduced-CP diets could be expected to increase acetyl-CoA concentrations which, in turn, could inhibit or downregulate hepatic TDH activity by selective feedback inhibition, resulting in elevated free threonine plasma concentrations. It is conceded that the proposed rationale is speculative. It is possible that lower dietary protein concentrations are contributing to elevated threonine plasma concentrations by depressing TDH activity; however, this mechanism may be subordinate to the proposed rationale. Nevertheless, it should be possible to assess the validity of the rationale by analysing hepatic acetyl-CoA concentrations (Shurubor *et al.* 2017) and TDH activity (Aoyama and Motokawa 1981) allied to threonine plasma concentrations in birds offered reduced-CP diets; certainly, this is our intention.

Insulin and glucagon

A mechanism whereby threonine plasma concentrations in birds offered reduced-CP diets has been tentatively identified. Nevertheless, this mechanism is not operating in a 'metabolic vacuum' and the conversion of glucose to acetyl-CoA is pivotal to the proposed mechanism. Plasma glucose concentrations are higher in avian than in mammalian species (15.3 versus 7.6 mM/L) by a two-fold factor (Braun and Sweazea 2008), but the biological basis for this difference has not been defined (Scanes 2009). Broiler chickens offered reduced-CP diets are effectively subject to a 'starch overload' and the resultant increased intestinal uptakes of glucose have an impact on the pancreatic secretion of insulin and glucagon, powerful hormones that have either anabolic or catabolic functions respectively, to the extent that molar ratios of insulin to glucagon have been described as determinants of avian carbohydrate metabolism (Hazelwood 1984). In addition, insulin has been shown to increase leptin

expression in liver, but not in adipose tissue, of chickens and, reciprocally, glucagon has been shown to decrease hepatic expression of leptin but not in adipose tissue (Taouis *et al.* 2001). However, avian species appear to be sensitive to glucagon but resistant to insulin (Braun and Sweazea 2008). The likelihood is that both insulin and glucagon affect the performance of birds offered reduced-CP diets, including elevated threonine plasma concentrations, compromised FCR and heavier relative fat-pad weights. Of relevance is that excessive fat deposition in rapidly growing broilers has been attributed to increased plasma concentrations of insulin and glucagon and, possibly, insulin resistance (Sinsigalli *et al.* 1987). In relation to starch and protein digestive dynamics, Selle and Liu (2019) concluded that a better delineation of the starch–glucose–insulin axis in avian physiology is necessary and this caveat equally applies to the reciprocal glucose homeostatic roles of insulin and glucagon in broiler chickens offered reduced-CP diets.

Threonine, glycine and serine

In theory, threonine is a precursor of glycine (Baker *et al.* 1972) and both TDH and threonine aldolase catalyse the enzymatic conversion of threonine to glycine and glycine and serine are inter-convertible in poultry (Sugahara and Kandatsu 1976). Although classified as non-essential amino acids, glycine and serine, or glycine equivalents, have been shown to be of critical importance in reduced-CP broiler diets, and Siegert and Rodehutsord (2019) recommended that glycine equivalent requirements fall within a broad range from 11 to 20 g/kg. However, the notable increases in plasma threonine concentrations do not indicate that threonine is being converted to glycine and, in turn, serine. Indeed, increasing threonine plasma concentrations quadratically depressed glycine plasma concentrations to significant extents in both Chrystal *et al.* (2020c), where $r = 0.486$ and $P = 0.036$, and Chrystal *et al.* (2020d), where $r = 0.632$ and $P = 0.002$. Increasing threonine plasma concentrations tended to depress glycine concentrations quadratically ($r = 0.514$, $P = 0.117$) when consideration was given to all the Table 1 data. This agrees with the proposal that conversion of threonine to glycine and acetyl-CoA by TDH is being downregulated and, as a consequence, threonine is not serving as a glycine precursor. In more general terms, this is consistent with the conclusion of D'Mello (1973) that threonine is not readily degraded to glycine and does not act as a glycine precursor.

Dietary starch:protein ratios

Typically, soybean meal inclusions are decreased, and maize increased, in the formulation of reduced-CP diets. This substitution achieves reductions in CP and then targeted amino acid requirements are met by increasing inclusions of an expanding array of non-bound amino acids. For example, soybean meal inclusions were decreased by 52.7% (157 versus 331 g/kg) and maize inclusions increased by 31.6% (732 versus 556 g/kg) collectively, with the transition from the highest- to lowest-CP diets in Chrystal *et al.* (2020b, 2020c,

2020d, 2021). Also, inclusions of non-bound amino acids increased from 5.8 to 26.7 g/kg to meet requirements. This resulted in an expansion of analysed dietary starch:protein ratios from 1.55 to 2.50 in these four studies. There is a quadratic relationship between analysed dietary starch:protein ratios with relative fat-pad weights ($r = 0.754$, $P = 0.006$) and a linear relationship with FCR ($r = 0.417$, $P = 0.096$) across the Table 1 studies; thus, expanding starch:protein ratios may have deleterious impacts on these critical parameters.

The biological validity of these statistically significant outcomes is problematic; nevertheless, they prompted an initial evaluation of the strategy of limiting or ‘capping’ expansions of starch:protein ratios in reduced-CP diets. Some promise was displayed in this evaluation by Greenhalgh *et al.* (2020b). Broiler chicks were offered 197.5 g/kg CP wheat-based diets with dietary starch:protein ratios of either 1.97 or 1.63 from 7 to 35 days post-hatch. Diets with the narrower dietary starch:protein ratio supported better growth performance with a significant improvement in weight gain of 10.4% (2161 versus 1958 g/bird) and a numerical improvement in FCR of 4.04% (1.616 versus 1.684). The partial or total substitution of soybean meal (475 g/kg CP) with full-fat soy (360 g/kg CP) is one approach where dietary CP could be reduced to some extent without an increase in dietary starch concentrations. The proposed rationale that glucose from dietary starch is downregulating TDH activity because it is a source of acetyl-CoA suggests that the strategy of capping expansions in dietary starch:protein ratios merits further investigations. The total replacement of soybean meal with full-fat soy in broiler diets would lower dietary CP by ~30 g/kg without increasing dietary starch concentrations. More tangible CP reductions would necessitate some increases in dietary feed grain inclusions and starch concentrations, but modest increases may be accommodated. If the strategy of capping dietary starch:protein ratios proves to be a viable approach, it is quite possible that elevations in threonine plasma concentrations would be moderated.

Maize versus wheat as a feed grain in reduced-CP diets

The feed grain component, usually maize or wheat, is increased at the expense of protein-rich feedstuffs, usually soybean meal, in reduced-CP diets. Axiomatically, this increases quantities of dietary starch and expands dietary starch:protein ratios. Moreover, as shown in Fig. 3, there is a quadratic increase ($r = 0.664$, $P = 0.007$) in free threonine plasma concentrations in response to expanding dietary starch:protein ratios from the Table 1 data. Given these increases in maize or wheat inclusions, the starch and protein concentrations of the feed grain assume more importance. Moreover, it does appear that the birds are better able to accommodate CP reductions of maize-based diets than of wheat-based diets (Chrystal *et al.* 2021). In this study, birds offered 165 g/kg CP maize-based diets significantly outperformed birds offered wheat based by 53.0% (2370 versus 1549 g/bird) in weight gain, 22.4% (3481 versus 2843 g/bird) in feed intake and by 19.9% (1.473 versus

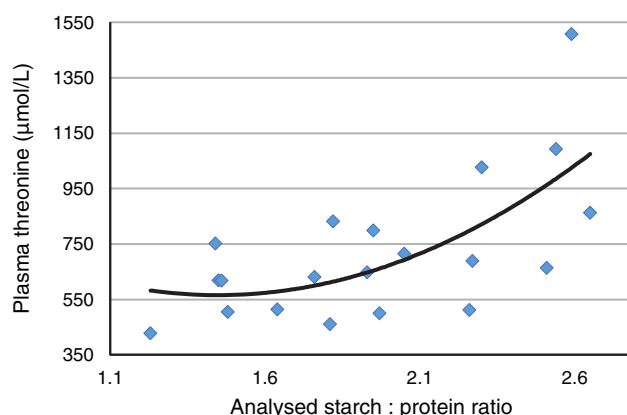


Fig. 3. Quadratic relationship ($r = 0.676$; $P = 0.014$) between analysed dietary starch:protein ratio (S:P) and free threonine concentrations in systemic plasma where $y_{(\text{thr } \mu\text{mol/L})} = 600 - 256.25 \times \text{CP}_{(\text{g/kg})} + 141.44 \times \text{CP}_{(\text{g/kg})}^2$.

1.840) in FCR. Interestingly, systemic plasma threonine concentrations were 75% (180 versus 103 μg/mL) higher in birds offered the wheat-based diet. Again, this is not to suggest that the difference in plasma threonine concentrations was causative, but quite possibly indicative, of the inferior growth performance supported by the wheat-based diet. The factors contributing to the relative inferiority of wheat need to be identified. The higher protein concentration of wheat results in more non-bound amino acids and less ‘intact’ soy protein in reduced-CP diets than is the case with maize. Also, there is the likelihood that wheat starch may be digested more rapidly than is maize starch, which is the case under *in vitro* conditions (Giuberti *et al.* 2012). These three factors are possibly involved in the inferiority of wheat in this context, which appears to be reflected in elevated threonine plasma concentrations.

Conclusions

In conclusion, tangible reductions in dietary CP usually compromise FCR, increase fat deposition and trigger elevations in free threonine systemic plasma concentrations. The elevated threonine concentrations are not thought to be causative; nevertheless, the likelihood is that they are indicative of a compromised performance. The strategy of limiting increases in starch concentrations in reduced-CP broiler diets may enhance performance. However, if the proposed mechanism whereby threonine dehydrogenase is downregulated is valid, elevations in free threonine plasma concentrations may be attenuated as a secondary effect. Finally, the likelihood is that free threonine plasma concentrations may be an indicative biomarker of the precision with which efficient reduced-CP broiler diets are formulated and, if so, would facilitate their successful development.

Conflicts of interest

The authors declare that there not any conflicts of interest.

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