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Non-ruminants

Individual responses of growing pigs to threonine intake

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ABSTRACT - A nitrogen balance test was performed to evaluate the individual responses of growing pigs to threonine intake. Eight commercial barrows were used (body weight ranging from 15 to 20 kg). A dose-response study was performed, in which the threonine supply increased in seven equidistant steps (the seven dietary threonine levels ranged from 50 to 120% of the requirements) every three days for each pig. The levels of all other amino acids were 20% higher than the tested amino acid. Nitrogen retention as a function of threonine intake was calculated per individual and per group (NLIN and NLMixed, respectively) using a linear plateau model. The highest break point value was 42.42 g of threonine intake (the most demanding individual), whereas the lowest value was 34.16 g (the least demanding individual), corresponding to a difference of 19%. In terms of N retention, the highest plateau value was 66.71 g and the lowest was 49.48 g, with a difference of 25%. There was no significant correlation between slope and plateau values or between slope and break point values. When using the model in which all parameters were random effects, the variations in threonine intake and nitrogen retention were 1.68±1.30 and 0.01±0.10 g, respectively, and no variance in the slope of the curve was detected. The average daily threonine intake values for the maximum response obtained in the group, as calculated by the NLIN and NLMixed procedures, were 13.96 and 14.02 g/day, respectively. The threonine intake for the maximum N retention between individuals ranged from 34.16 to 42.42 g, corresponding to a difference of 19%. The current recommended intake to optimize N retention is 14.02 g/day. The group responses obtained by the NLMixed procedures are very similar to those estimated by the NLIN procedure (all individuals).

Key Words: amino acids, individuals, nitrogen retention, requirements, variability

Introduction

Threonine is considered an essential amino acid (AA) and is commonly the second or third limiting AA in pig diets based on corn and soybean meal; however, it may be the first limiting AA when diets are supplemented with synthetic lysine (Saldana et al., 1994). Threonine is critical for maintenance because it is used for the synthesis of muscle protein, mucin in the gastrointestinal system, and immunoglobulins (Nichols and Bertolo, 2008).

An increasing number of studies attempting to determine threonine requirements have been published (Rostagno et al., 2011; NRC, 2012) and the main methods applied are dose-response, which are based on the average response of the population, or factorial, which are based on the response of an average individual. These methods allow only a single nutrient level to be established and supplied to all animals in a given category for a long period and do not consider the dynamics of the requirements and the variability among individuals within a population (Hauschild et al., 2010). This variability is explained by intrinsic animal factors, such as body weight, genotype, age, and sex, and by extrinsic factors, such as environmental temperature and animal density. Those factors influence the determination of the optimal requirement of a specific nutrient and are essential for understanding the biological mechanisms involved in the responses of the population to this nutrient (Pomar et al., 2003). In addition, the variability among individuals within a population must be determined to optimize the biological, economic, and environmental responses and to establish more accurate nutritional programs.

Some studies have reported a significant influence of the variability among individuals within a population on the performance responses and on the nutritional requirements of growing pigs (Ferguson et al., 1997; Bertolo et al., 2005; Moehn et al., 2008). Although some studies accounted for that variability, few papers have been published aiming to evaluate the effect that the variation among individuals

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has on AA requirements (Pomar et al., 2003). In addition, when estimating animal responses to different intake levels of a nutrient, variability should be considered to increase the precision of these estimates. Therefore, the objective of the present study was to evaluate individual and group responses of growing pigs to dietary threonine supply by using a sequential short-term N balance method.

Material and Methods

The experimental procedures were reviewed and approved by the institutional committee on animal use (case no. 018435/13). The experiment was carried out in Jaboticabal, São Paulo, Brazil (21°15'16" S, 48°19'19" W, and 607 m altitude).

Eight barrow pigs (18.83±0.28 kg body weight) of the same high-performance genotype (Camborough × GPIC337; Agroceres PIC Inc.) with good health status were shipped in a single batch to a swine research facility in Jaboticabal, Brazil. All pigs were housed in metabolic cages in a room under controlled temperature (22 to 24 °C) and allowed to adapt to their new environment for eight days before the start of the experiment. Animals were weighed at the beginning and end of the experimental period.

During the adaptation period, pigs were fed a diet formulated to supply their nutritional requirements. In the last three days of the adaptation period, the diet contained 50% less threonine for adaptation to the experimental diets. Subsequently, a 21-day dose-response study was performed, in which the threonine supply increased in seven equidistant steps every three days for each pig, following Kampman-van de Hoek et al. (2013). Threonine levels ranged from 50 to 120% of the requirements (Rostagno et al., 2011). For this purpose, one concentrated diet was formulated to contain 122% of the digestible threonine requirements of pigs (Table 1) and all other amino acids were added at levels 20% higher than the threonine requirement to maintain their ideal ratio to lysine, according to Rostagno et al. (2011). This diet was sequentially diluted with a diet based on starch and free from all amino acids (diluent diet) (Table 1) to obtain the experimental diets containing the seven digestible threonine levels proposed (Table 2). Both diets contained equal levels of energy.

Pigs were offered the same amount of feed twice daily, at 8.00 and 14.00 h. The amount of feed supplied daily to each individual animal was calculated as 2.6 times its maintenance energy requirement (250 kcal metabolizable energy kg^{-1} live $weight^{0.60}$), according to Noblet and Shi (1993). Pigs were offered water *ad libitum* throughout the experimental period.

Faeces were collected at 8.00 h for three consecutive days for each dietary threonine level. At the end of the third day, the faeces collected during the three days were

Table 1 - Ingredients and chemical composition of experimental diets

Item	Concentrated	Diluent
Ingredient (g kg^{-1} dry matter)		
Corn (78.8)	697.3	-
Soybean meal (450.0)	126.7	-
Corn starch	-	850.0
Rice husk	53.6	101.0
Soybean oil	29.1	23.4
Dicalcium phosphate	14.2	18.0
Limestone	4.8	2.6
Sodium bicarbonate	0.8	0.8
Salt	2.1	2.9
Mineral and vitamin premix[1]	2.0	2.0
Butylate toluene hydroxide	0.1	0.1
L-alanine	19.3	-
L-lysine HCl (780.0)	15.1	-
L-valine	6.5	-
L-leucine	6.5	-
L-threonine	5.8	-
L-isoleucine	5.1	-
L-phenylalanine	3.3	-
Dl-methionine	3.2	-
L-histidine	2.6	-
L-tryptophan	2.0	-
Calculated composition		
Crude protein[3] (g kg^{-1} dry matter)	178.2	5.3
Metabolizable energy[2] (kcal kg^{-1})	3.30	3.30
Calcium[3] (g kg^{-1} dry matter)	6.3	6.3
Disponible phosphorus[2] (g kg^{-1} dry matter)	3.3	3.3
SID lysine[3] (g kg^{-1} dry matter)	17.0	-
SID methionine[3] (g kg^{-1} dry matter)	4.9	-
SID threonine[3] (g kg^{-1} dry matter)	9.4	-
SID tryptophan[3] (g kg^{-1} dry matter)	3.1	-
SID valine[3] (g kg^{-1} dry matter)	11.9	-
SID isoleucine[3] (g kg^{-1} dry matter)	9.9	-

SID - standard ileal digestible.
[1] Mineral and vitamin mixture (content per kg of supplement): folic acid, 350 mg; selenium, 75 mg; copper, 9,000 mg; calcium pantothenate, 6,000 mg; biotin, 10 mg; manganese, 25,000 mg; iodine, 125 mg; cobalt, 125 mg; niacin, 14,000 mg; zinc, 48,000 mg; iron, 48,000 mg; vitamin A, 3,500,000 IU; vitamin B1, 400 mg; vitamin B12, 11,000 µg; vitamin B2, 1,600 mg; vitamin B6, 500 mg; vitamin D3, 500,000 IU; vitamin E, 5,000 IU; vitamin K, 1,000 mg; antioxidant (BHT), 2,000 mg.
[2] Composition of nutritional ingredients proposed by Rostagno et al. (2011).
[3] Determined through high-performance liquid chromatography analysis.

Table 2 - Mixture of diets to obtain threonine levels

Composition[1]	Level of digestible SID threonine (%)						
	50	62	74	86	98	110	122
Concentrated diet[2]	409.8	507.8	606.5	704.9	802.7	901.3	1000.0
Diluent diet[2]	590.2	492.2	393.5	295.1	197.3	98.7	0.0
Crude protein[2]	77.5	90.8	104.7	125.8	141.2	156.5	178.2
SID lysine[2]	7.6	9.6	10.2	12.5	14.2	15.8	17.0
SIS methionine[2]	2.1	2.8	3.2	3.7	4.2	4.7	4.9
SID threonine[2]	4.1	5.5	6.2	7.1	8.0	8.5	9.4
SID tryptophan[2]	1.4	1.7	1.9	2.3	2.5	2.7	3.1
SID valine[2]	5.1	6.5	7.4	8.8	9.6	10.8	11.9
SID isoleucine[2]	4.2	5.5	6.3	7.2	8.0	8.8	9.9

SID - standard ileal digestible.
[1] Determined through high-performance liquid chromatography analysis.
[2] g kg^{-1} dry matter.

pooled, placed in a plastic bag, with the individual pig identified, and stored in a freezer at −10 °C. At the end of the collection period, faeces were homogenized and a 0.5 kg aliquot was taken, dried in a forced-ventilation oven (60 °C for 72 h), and ground in a ball mill for subsequent chemical analyses.

Urine was collected in plastic buckets, containing 25 mL of HCl, at 8.00 h for three consecutive days for each dietary threonine level. The urine volume was measured and homogenized and a 5% aliquot was collected every 24 h. Urine samples were stored in a refrigerator at 4 °C. At the end of the third day, urine samples were pooled in each threonine level for subsequent analyses. Samples of each diet were randomly collected and analysed for total amino acid composition. The results showed that the analysed values were consistent with the calculated values. Amino acid composition was determined by high-performance liquid chromatography. The dry matter content of the diets and faeces was determined according to the AOAC (2005). The total N content of the diets, faeces, and urine was determined by the combustion method in a LECO/FP-528 apparatus.

Based on apparent N digestibility estimated by the indicator method, the daily faecal N excretion was calculated. Daily N retention was calculated as N intake at day (i) minus urinary N losses and faecal N losses at day (i+1). The threonine intake was calculated as the percentage of threonine in the diet multiplied by the feed intake (g). The obtained data were fit to a linear response plateau (LRP) model to evaluate N retention as a function of threonine intake and were then subjected to residual analysis to determine the goodness of fit of the models.

Two methodologies of analysis were applied in this study. In the first methodology, data were evaluated per individual animal and per group using the NLIN procedure of SAS (Statistical Analysis System, version 9.3). The LRP model described the N retention response (NR) as a function of threonine intake (Thr_{int}) according to the following equation:

$$NR = NR_p + b\,(I - Thr_{int}) \text{ when } I < Thr_{int} \text{ and}$$
$$NR = NR_p \text{ when } Thr_{int} \geq I,$$

in which NR_p is the plateau, b is the slope of the ascending line, I is the break point of the threonine intake response, and Thr_{int} is the dietary threonine intake.

The second methodology used to analyse the data was the NLMixed procedure of SAS, which uses sequential analyses (four steps or models) to evaluate the group response, considering the individual variability. First, the LRP model was fitted for each individual response to determine the initial parameters to use in the subsequent analyses. The first model, considering the total set of experimental animals, was generated with fixed effects. Subsequently, the random parameters u_I, u_b, and u_{NR} were then created to account for the variation among individuals and to estimate the parameters I (break point), b (slope), and NR_p (plateau). The random effects u_I, u_b, and u_{NRp} were progressively added to the subsequent models. Therefore, the second model considered the random effect u_I, the third model considered the effects u_I and u_b, and the fourth model included the effects u_I, u_b, and u_{NRp}. This model was applied using the NLMixed procedure of SAS based on Wolfinger (1999). The steps that included the random effects were analysed using the FIRO (First Order) method and considered the individual animal (replicate) as a factor of fit. The proximity of fit of the four models used in the NLMixed procedure was evaluated by comparing the residual mean squares of each individual using the Wilcoxon paired test.

Results

Animals presented good health status, as indicated by a lack of diarrhoea and their observed feed intake being similar to the estimated feed intake, with the exception of one animal that died during the experiment due to causes unrelated to the feeding program or methodology applied. The average body weight at the beginning of the experiment was 22.14±0.90 kg and the final body weight (after 21 days) was 34.59±2.44 kg (Table 3), resulting in an average daily gain of 0.59 kg.

The average Thr intake and N balance for pigs increased when dietary Thr concentration increased (Table 4). The coefficients of determination of the LRP model analysed by the NLIN procedure applied to each individual animal ranged between 0.92 and 0.99, indicating a goodness of fit (Table 5). The coefficient of variation of the parameter related to N retention (NRp) was 5.2%.

Table 3 - Mean values of initial and final body weight (BW) for pigs fed different levels of threonine

Animal	Initial BW (kg)	Final BW (kg)
1	21.80	39.07
2	23.33	33.61
3	20.83	32.73
4	23.02	34.44
5	22.09	36.75
6	22.59	33.16
7	21.34	32.41
Mean	22.14	34.59
CV	4.06	7.07

CV - coefficient of variation.

In the LRP model generated by the NLIN procedure for all animals (Figure 1), threonine intake was estimated as 41.89 g (break point). The highest break point value was 42.42 g of threonine intake for the individual that was the most demanding, whereas the lowest value was 34.16 g for the individual that was the least demanding, corresponding to a threonine intake difference of 19% for the maximum response. In terms of N retention, the highest plateau value was 66.71 g and the lowest was 49.48 g, representing a difference of approximately 25%. There was no significant correlation between the slope and plateau values (r = −0.34; P = 0.41) (Table 5) or between the slope and break point values (r = 0.02; P = 0.97). However, a positive correlation between the break point and plateau values was detected (r = 0.72; P = 0.04).

No significant difference (P>0.05) was determined when the fit of the four models applied were compared (models 1-2, P = 1.00; models 1-3, P = 0.93; models 1-4, P = 0.93; models 2-3, P = 1.00; models 2-4, P = 1.00; and models 3-4 P = 1.00; Table 6). The analysis of the data using the NLMixed procedures allowed the variance among individuals to be evaluated for each parameter. The first model considered the entire set of experimental animals and was generated with fixed effects; therefore, there was no parameter variation among individuals. The second model added the break point as a random effect and the threonine intake variance among individuals was determined as 3.11±1.76 g. The parameters for break point and slope were added as random effects in the third model. The break point presented a variance in threonine intake of 1.61±0.61, whereas no variance due to slope was detected. The fourth and last model yielded break point and plateau values of 1.68±0.63 and 0.01±0.03, respectively, but no variation was detected in the slope values.

The group responses obtained by the NLMixed procedures were very similar to those estimated by the NLIN procedure (all individuals). The average daily threonine intake values for maximum response obtained by the NLIN and NLMixed procedures were 13.96 and 14.02 g/day, respectively.

Table 4 - Mean values of nitrogen balance (N) in pigs fed different levels of threonine[1]

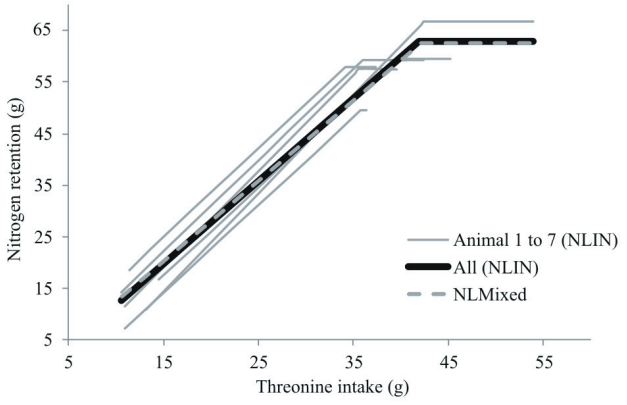
Threonine level (%)	Threonine intake (g)	N intake (g)	Urinary N (g)	Fecal N (g)	Retained N (g)
50	11.92	39.01	9.53	16.58	12.90
62	17.32	53.89	9.73	19.17	24.99
74	22.09	66.28	10.65	23.86	31.77
86	27.70	82.50	12.30	31.58	38.63
98	32.41	95.99	16.10	33.30	46.60
110	36.43	111.66	16.53	36.58	58.55
122	42.58	128.48	27.27	42.69	58.52
SEM	4.10	12.10	2.37	3.61	6.46

[1] Average values correspond to the period of the three days of each diet.
SEM - standard error of the mean.

Table 5 - Parameters of linear-plateau models relating N retention (NR) to threonine intake (Thr_{int}), calculated values of the slope (b_0), break point (I), and plateau (NRp)[1,2], and correlations between model parameters

Animal	Model parameter			R^2
	b	*I*	*NRp*	
1	−1.910	42.424	66.708	0.95
2	−1.776	35.926	59.321	0.99
3	−1.701	39.742	59.622	0.94
4	−1.704	35.732	49.482	0.97
5	−1.560	39.991	59.274	0.97
6	−1.579	38.600	57.536	0.92
7	−1.729	34.155	57.959	0.94
All	−1.606	41.889	62.971	0.91
	Correlation between model parameters			
	b	*I*	*NRp*	
b	1.00	0.34	−0.34	
I	-	1.00	0.72*	
NRp	-	-	1.00	

[1] $NR = NRp + b\ (I - Thr_{int})$ when $I < Thr_{int}$ and $NR = NRp$ when $Thr_{int} \geq I$.
[2] Values correspond to the period of the three days of each diet.
*Significant at 5%.



Values correspond to the period of the three days of each diet.
LP for all individuals [NR = 62.971 − 1.606 (41.889 – Thr_{int}) when 41.889 < X and NR = 62.971 when $Thr_{int} \geq 41.889$ ($R^2 = 0.91$)].
LP animal 1 [NR = 66.708 − 1.910 (42.424 – Thr_{int}) when 42.424 < X and NR = 66.708 when $Thr_{int} \geq 42.424$ ($R^2 = 0.95$)].
LP animal 2 [NR = 59.321 − 1.776 (35.926 – Thr_{int}) when 35.926 < X and NR = 59.321 when $Thr_{int} \geq 35.926$ ($R^2 = 0.99$)].
LP animal 3 [NR = 59.622 − 1.701 (39.742 – Thr_{int}) when 39.742 < X and NR = 59.622 when $Thr_{int} \geq 39.742$ ($R^2 = 0.94$)].
LP animal 4 [NR = 49.482 − 1.704 (35.732 – Thr_{int}) when 35.732 < X and NR = 49.482 when $Thr_{int} \geq 35.732$ ($R^2 = 0.97$)].
LP animal 5 [NR = 59.274 − 1.560 (39.991 – Thr_{int}) when 39.991 < X and NR = 59.274 when $Thr_{int} \geq 39.991$ ($R^2 = 0.97$)].
LP animal 6 [NR = 57.536 − 1.579 (38.600 – Thr_{int}) when 38.600 < X and NR = 57.536 when $Thr_{int} \geq 38.600$ ($R^2 = 0.92$)].
LP animal 7 [NR = 57.959 − 1.729 (34.155 – Thr_{int}) when 34.155 < X and NR = 57.959 when $Thr_{int} \geq 34.155$ ($R^2 = 0.94$)].

Figure 1 - Individual N retention (NR) in relation to threonine intake using the Linear Plateau model (LP) and the mixed model.

Table 6 - Parameters generated by the NLMixed procedure between nitrogen retention (NR) and threonine intake (Thr_{int}), calculated values of slope (b), break point (I), plateau (NRp), and variances (u_I, u_b, and u_{NRp}) of parameters[1]

Model	b	I^2	NRp^2	u_I^2	u_b^2	u_{NRp}^2
1 - Fixed effects	−1.606	41.889	62.978	-	-	-
2 - Random effects (u_I)	−1.611	41.914	62.971	3.111	-	-
3 - Random effects (u_I, u_b)	−1.564	42.072	62.691	1.610	0.000	-
4 - Random effects (u_I, u_b u_{NRp})	−1.568	42.072	62.691	1.681	0.000	0.010

[1] Mean values corresponding to the period of the three days of each diet.
[2] $NR = NR + b(I - Thr_{int})$ when $I_0 < Thr_{int}$ and $NR = NRp$ when $Thr_{int} \geq I$.

Discussion

The slopes of the LRP models were different among individuals, showing that there was variability between them. However, there was no correlation between the slope and the plateau, suggesting that protein deposition was not associated with the efficiency of amino acid utilization in the animals evaluated. However, a correlation was observed between the broken line and plateau parameters, which suggested that protein deposition was associated with the threonine intake. In fact, this correlation was expected. Moehn et al. (2004) used indicator amino acid oxidation methodology to estimate amino acid catabolism and concluded that animals with high protein deposition potential present lower lysine catabolism, with a consequently better efficiency in utilizing this amino acid. This indicates a positive correlation between the efficiency of lysine utilization and the protein deposition potential of the animal. The absence of an observed correlation in our study and in assays using similar methodology may be related to the empirical form of representing efficiency, particularly to the mathematical interpretation that is used to fit individual response (i.e., parameters are independently estimated).

The 5.2% coefficient of variation is consistent with the findings of published studies about the response of growing pigs to amino acid intake (Heger et al., 2007; Heger et al., 2008; Heger et al., 2009). This indicates that the maximum protein deposition rate also varies considerably among individuals, even when animals present homogenous characteristics (genetic strain, age, and sex) and are evaluated under the same environmental and health-challenged conditions. In addition, it emphasizes the importance of pre-selecting animals according to their protein deposition potential (De Lange et al., 2001), particularly when experiments are designed with a limited number of animals per treatment.

The mean daily values of threonine intake for the maximum N retention obtained by NLIN and NLMixed procedures were higher than the daily values recommended by the NRC (2012); the recommendation for threonine is 9.15 g/day for pigs weighing between 20 and 50 kg. However, for animals with a high potential for lean meat deposition, the threonine requirements increase. Thong and Liebert (2004) observed that the threonine recommendation can vary between 8.96 and 12.22 g/day for pigs that have lean meat deposition between 130 and 160 g/day, respectively.

One of the reasons that can help explain why there were no observed differences between models is that the threonine intake variance among individuals was observed only for the break point parameter. For the slope parameter, no variance was detected. This can also be due to the empirical form of representing efficiency (slope) that we used to fit individual response. This form of efficiency assumes no differences among individuals for feed efficiency. Furthermore, the variance for break point was lower. The low variability among the pigs in this study may have contributed to this result. Despite of that, it is important to observe that, even in homogenous populations, some important variability can be detected (e.g., a 5% coefficient of variation in the threonine intake). Consequently, when a group of pigs is heterogeneous (i.e., when there is higher variability among individuals), the results obtained with this methodology may be more appropriate. The method based on the NLMixed procedure may provide more accurate estimates of the requirements for maximum response (i.e., protein deposition) because it allows the inclusion of other parameters (e.g., break point) to be included as random effects in the analysis.

One of the main problems when performing dose-response studies with growing pigs is the duration of the experimental period. Nitrogen balance techniques typically requires five to seven days of supply of each studied amino acid level. Obviously, the physiological status of the animals was not identical during the experimental period. In the present study, the method of supplying diets with increasing threonine levels every three days may have overcome this issue because the urinary N content reaches a new balance within three days after the dietary addition of the amino acid, which limited protein deposition (Brown and Cline, 1974; Fuller et al., 1979). Therefore, this technique may

be used to study the response of individual animals to amino acid intake and, consequently, the variability of their nutritional requirements.

The importance of considering the variability among individual animals has been noted for several decades, but few studies have evaluated its effects on amino acid requirement estimates (Pomar et al., 2003). The method used to evaluate individual responses in the present study showed that there is individual variation in N retention as a function of threonine intake. Because the fitting method of the NLMixed procedure included the random effects in a gradual manner, it allowed the variability in threonine intake and N retention within each group to be quantified, which contrasts previous studies (Saldana et al., 1994; Coma et al., 1995) that only applied methods that considered the average response of the population.

The individual nitrogen retention responses to the threonine intake levels obtained in this study provided relevant information on the variability of individuals, which may be used to improve stochastic models and develop more accurate nutritional and genetic strategies.

The sequential short-term N balance method gave values for threonine requirements that were similar to conventional methods. The short experimental period allows the estimation of population variability, which provides a more accurate calculation of the effect of altering threonine intake on herd performance. When estimating the optimal levels for a group, the variations in individual response should be considered, but the method based on the NLMixed procedure provides more accurate estimates because it allows the inclusion of random effects in the analysis.

Conclusions

The current recommended threonine intake to optimize N retention is 14.02 g/day. The group responses obtained by the NLMixed procedures are very similar to those estimated by the NLIN procedure (all individuals).

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