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RESEARCH PAPER
https://doi.org/10.1071/AN22241

ANIMAL PRODUCTION SCIENCE

Effects of dietary conjugated linoleic acid in broiler breeders and egg storage time on the fatty acid profile, lipid oxidation and internal egg quality

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Handling Editor:
Kris Angkanaporn

Received: 7 July 2022
Accepted: 2 May 2023
Published: 2 June 2023

Cite this:
Silvério PS *et al.* (2023)
Animal Production Science, **63**(12), 1208–1214.
doi:10.1071/AN22241

ABSTRACT

Context. The need for the storage of fertile eggs is a reality in the poultry industry. At the same time, prolonged storage periods decrease the quality of egg components that are essential for embryo development, and can compromise hatchability and chick quality; thus, the high content of unsaturated fatty acids in embryo tissues increase the susceptibility to peroxidation. **Aims.** The objective of this study was to evaluate the addition of *cis*-9, *trans*-11, *trans*-10 and *cis*-12 isomers of conjugated linoleic acid (CLA) to the broiler-breeder diet and the storage time on the internal egg quality, composition and lipid oxidation. **Methods.** In total, 22 000 Cobb female broiler breeders of 58 weeks of age were fed with diets containing 0 or 0.024% CLA and fertile eggs were stored 3, 6 or 9 days prior to incubation. In total, 6912 hatching eggs were used in a completely randomised experimental design in a 2 × 3 factorial arrangement (CLA inclusion × egg storage time). At the end of each storage period, 30 eggs per dietary treatment were sampled to analyse yolk and albumen height, percentage and pH, yolk:albumen ratio, yolk diameter and index, Haugh unit (HU), yolk lipid oxidation, acidity and fatty acid profile. **Key results.** The progression of storage negatively affected the internal quality of the eggs; however, inclusion of CLA minimised these effects up to Day 6, especially for yolk diameter, HU, height and albumen pH. The total lipid content was not affected by the dietary treatments; however, CLA inclusion resulted in a higher proportion of stearic acid and a lower concentration of linoleic acid in yolks. **Conclusions.** The changes observed in fatty acid profile of the eggs may have favoured the reduction of lipid oxidation, as shown by the decrease in the acidity index and thiobarbituric acid-reactive substance (TBARS) values at shorter storage periods. **Implications.** The dietary addition of CLA to broiler breeders may be used to preserve the egg internal quality during a short-term storage period.

Keywords: conjugated linoleic acid, egg storage, Haugh unit, lipid profile, poultry nutrition, poultry production, TBARS, yolk acidity index.

Introduction

Conjugated linoleic acid (CLA) is a group of positional geometric isomers of linoleic acid with *cis*-9, *trans*-11, *trans*-10 and *cis*-12, considered as the most biologically relevant (Aydin and Cook 2009), found mainly in foods derived from ruminants (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) 2016), or can be chemically synthesised through the isomerisation of linoleic acid (Banni 2002).

Mainly known for its antioxidant activity (Stangle 2000; Du *et al.* 2001; Joo *et al.* 2002; Ko *et al.* 2004), and anticancer (Basu *et al.* 2000; Kilian *et al.* 2002) and body fat-reducing (Leung and Liu 2000; Park and Pariza 2007) effects, CLA can be an alternative for poultry industry to increase broiler productivity and the quality of day-old chicks.

It is important to consider that the addition of CLA to the breeder's diet may modify the size of the eggs, yolk and albumen (Suksombat *et al.* 2006), change the fatty acid profile of

the yolks (Suksombat *et al.* 2006; Aydin and Cook 2009) and thereby affect hatchability, causing an increase in embryo mortality (Muma *et al.* 2006).

Likewise, the storage of fertile eggs in the poultry industry is a reality. However, prolonged storage periods decrease the quality of egg components that are essential to embryo development (Barbosa *et al.* 2008) and can compromise hatchability (Macari *et al.* 2013) and chick quality (Reijrink *et al.* 2010). Thus, the high content of unsaturated fatty acids in embryo tissues increase the susceptibility to peroxidation (Ko *et al.* 2004); therefore, it is important to search for antioxidant alternatives for long-term-stored eggs. It is well known that long storage periods can lead to lower hatchability (Fasenko 2007), slower embryo growth and increased mortality (Fasenko *et al.* 2001; Fasenko 2007).

The aim of this study was to evaluate the effects of dietary addition of CLA to broiler breeders associated with storage time on egg quality, composition and lipid oxidation.

Materials and methods

All experimental procedures were approved by the Ethics Committee at Brasília University (CEUA-UnB number 62528/2015), and followed Ethical Principles for the Use of Experimental Animals of the Brazilian Society of Science in Laboratory Animals (SBCAL/COBEA).

In total, 22 000 Cobb broiler breeders in the laying phase (58 weeks of age) were distributed equally into two dietary treatments (with CLA and without CLA) in two different sheds. Breeders were fed *ad libitum* with a diet (Table 1) formulated according to Rostagno *et al.* (2011) and supplemented (or not) with 0.024% CLA, which corresponded to the inclusion of 0.042% of the commercial product Lutalin during 29 days. The commercial product used was composed of 56% of methyl esters of CLA (C18:2), 28% of the *cis*-9, *trans*-11 isomers and 28% of the *trans*-10, *cis*-12 isomer, according to information from the manufacturer (BASF).

Freshly collected eggs (3546 eggs) from each dietary treatment (with or without CLA), selected and classified by weight between 63 and 72 g (total of 7092 eggs for the entire experiment), were divided into three groups to be stored at the egg room (average temperature and relative humidity were 19–21°C and 76% respectively) during 3, 6 or 9 days. Each group contained 1182 eggs and included randomly distributed trays, using a 2 × 3 factorial design (with or without CLA in the broiler breeder diet × 3 storage periods, namely, 3, 6 and 9 days).

Internal egg quality

At the end of each storage period, a sample of 30 eggs from each treatment (each egg was considered a repetition) was collected for quality measurements in the laboratory. The eggs were weighed individually with a scale (GEHAKA, model BK3000, São Paulo/SP, Brazil) and opened on glass boards to assess the height of the albumen and the yolk by using a tripod micrometer, as follows: albumen and yolk height (AH, YH), yolk diameter (YD), yolk and albumen pH (YpH, ApH), yolk weight (YW) for the yolk index (YI) and Haugh unit (HU), considering the following: $HU = 100 \log(h - 1.7\, w^{0.37} + 7.6)$, where h = height of the albumen (mm) and w = weight of the egg (g) (Brant and Shrader 1958). The yolks were manually separated and weighed individually. Albumen weight (AW) was calculated as follows: egg weight – (yolk weight + shell weight). For the yolk index (YI), the

Table 1. Ingredients and nutrient composition of the experimental diets.

Item	With CLA (%)	No CLA (%)
Ingredients		
Corn, 7.88% CP	68.179	68.128
Soybean meal, 45.5% CP	17.416	17.425
Limestone, 38% Ca	7.876	7.876
Kaolin	4.000	4.000
Meat and bone meal, 43% CP	1.500	1.500
Salt	0.223	0.223
Dicalcium phosphate	0.200	0.200
Sodium bicarbonate	0.150	0.150
Mineral premix[A]	0.150	0.150
Vitamin premix[B]	0.100	0.100
Choline 60%	0.082	0.082
DL-Methionine 88%	0.080	0.080
L-Threonine 98%	0.030	0.030
Antioxidants[C]	0.016	0.016
CLA[D]	–	0.042
Phytase[E]	0.003	0.003
Nutrient composition		
Metabolisable energy (kcal/kg)	2736	2734
Crude protein (%)	13.90	13.90
Digestible lysine (%)	0.60	0.60
Digestible methionine + cysteine (%)	0.47	0.46
Digestible threonine (%)	0.50	0.50
Calcium (%)	3.29	3.29
Available phosphorus (%)	0.20	0.20
Na (%)	0.34	0.34

[A]Mineral premix: manganese 80 000 mg, zinc 70 000 mg, iron 40 000 mg, copper 8000 mg, iodine 1000 mg.
[B]Vitamin premix: vitamin A, 14 000 IU; vitamin D3, 3000 IU; vitamin E, 110 000 mg; vitamin K3, 6000 mg; vitamin B1, 3000 mg; vitamin B2, 12 000 mg; vitamin B6, 6000 mg; vitamin B12, 30 mg, pantothenic acid, 20 000 mg; nicotinic acid, 60 000 mg; folic acid, 4000 mg; biotin 300 mg.
[C]Ethoxyquin 66.6%, BHA 99% and citric acid 99.5%.
[D]CLA (Lutalin).
[E]Phytase (Natuphos10 000 FTU).

formula used was: YI = h/d, where h = yolk height (mm) and d = yolk diameter (mm). The percentages of yolk (Y%) and albumen (A%) were calculated from their respective weights, divided by the egg weight, and multiplied by 100. The yolk:albumen ratio (Y:A%) was calculated according to the formula Y:A% = (yolk weight/albumen weight) × 100.

The albumen and yolk pH were evaluated in triplicate in a pool of five eggs by using a portable digital pH meter (Testo, model T 205, Lenzkirch, Germany). Then, albumen samples were frozen and kept in a domestic freezer (−14°C), the yolk samples were frozen for 24 h and freeze-dried (LIOTOP, Model L101, São Carlos/SP, Brazil) to minimise lipid oxidation damage. After freeze-drying, the samples were ground in a knife mill equipped with a cooling system (TECNAL, TE 631, Piracicaba/SP, Brazil) and stored until analysis.

Lipid oxidation in yolk

The assessment of lipid oxidation used the acidity index (AI) and the quantification of secondary lipid oxidation compounds by using TBARS. The acidity index (Ca 5a-40 method; Association of Official Analytical Chemists (AOAC) 1990) was performed in duplicate in a pool of five eggs; lipids extracted from yolks according to Folch *et al.* (1957) adapted by Bligh and Dyer (1959) and expressed in mg of NaOH/g of lipids. TBARS were evaluated in duplicate by using the method described by Sørensen and Jørgensen (1996), expressed in μmol of malonaldehydes (MDA)/kg of yolk.

Lipids and fatty acid profile in yolk

The total lipids (TL, % of raw matter) were evaluated using the AOAC methodology (Association of Official Analytical Chemists (AOAC) 1990), with six replicates per treatment, in a pool of five eggs in ground, freeze-dried yolk samples.

The fatty acid profile was determined in the same lipid samples extracted as mentioned for acidity index determination, followed by methylation (Christie 1989). The quantification of esterified fatty acids used a gas chromatograph (Shimadzu, CG-2014, Kyoto, Japan) with detector MS-QP2010 Plus and autoinjector AOC-5000. The separation of fatty acids was undertaken using a 60 m (length), 0.25 mm ID (internal diameter), 0.25 μm (film thickness) column from J & W Scientific (122-2362 DB-23). Helium was used as the carrier gas, with a continuous flow of 0.40 mL/min in the column. The injected volume was 1 μL (Split mode) and the detector temperature was 260°C. The heating conditions of the column were as follows: 140°C for 5 min, then increasing by 2°C every minute until 240°C, totalling 56 min of the chromatographic run. The identification of fatty acids was undertaken by comparing the peaks with the retention time of the standard fatty acid Supelco 37 component FAME mix (Supelco Analyticals™ from Merck Group, Darmstadt, Germany) and the results were expressed as a percentage of the area of each fatty acid, in relation to the total area.

Statistical analyses

The results were compared using a mixed model, with treatments being fixed effects and storage periods random effects, using PROC MIXED procedure from Statistical Analysis Systems (ver. 9.3; SAS Institute Inc 1989). The averages were compared by the Tukey test, with 5% significance.

Results and discussion

The yolk weight (YW) was not affected by the addition of CLA or by the storage time in the egg room (Table 2). Likewise, there was no effect of inclusion of CLA on yolk height (YH), which was negatively affected by storage time, as expected, and previously described (Oliveira and Oliveira 2013). Although storage time negatively affected the yolk index (YI), the addition of CLA preserved YI, as described by Shinn *et al.* (2015).

The addition of CLA did not affect the yolk percentage (Y%) and the yolk:albumen ratio (Y:A%); however, the increasing storage time raised these values, which can be explained by the decrease of the albumen weight (AW) in this period, since YW was not affected by treatments. This decrease of the albumen weight (AW) may have been caused by the difference in osmotic pressure that is responsible for the water movement from the albumen to the yolk during storage; thus, Y% increases and A% decreases, resulting in yolk enlargement and reduced viscosity (Macari *et al.* 2013).

The YpH was not affected by the dietary use of CLA. However, the storage caused an increase in YpH, as previously shown by Ganeco *et al.* (2012). However, Shinn *et al.* (2015) observed pH changes in eggs from breeders fed CLA only when stored up to 30 days, and it seems that higher changes in egg pH were associated with higher concentrations of CLA in the layer diets.

The storage time affected the yolk diameter (YD), as can be seen in Table 2, with YD increasing with the storage time, reaching the highest values at 9 days of storage (Table 2), since YD increases in liquefied yolk (Macari *et al.* 2013). In contrast, treatment with CLA preserved the YD until Day 6; however, this effect disappeared after 9 days of storage due to the antioxidant effect of dietary CLA described previously by Stangle (2000), Du *et al.* (2001), Joo *et al.* (2002) and Ko *et al.* (2004). The inclusion of CLA did not affect albumen weight (AW), which was reduced after 9 days of storage (Table 2) and can be explained by the increase in pH due to the loss of carbonic gas through the pores of the eggshell, causing liquefied albumen and decreased height (Karoui *et al.* 2006). Contrary to previous studies (Suksombat *et al.* 2006; Cherian *et al.* 2007), the dietary inclusion of CLA for breeders showed a positive effect on AH, preserving albumen quality, when compared with the control group. In turn, increasing storage time reduced AH, as expected. The albumen

Table 2. Effects of CLA in breeder diets and storage time in the egg room on average values of yolk weight (YW, g), yolk diameter (YD, mm), yolk height (YH, mm), yolk index (YI), percentage of yolk (Y%), yolk:albumen ratio (Y:A%), yolk pH (YpH), albumen weight (AW, g), albumen height (AH, mm), percentage of albumen (A%), Haugh unit (HU) and albumen pH (ApH), significance of main effects and interactions.

Factor	YW	YD	YH	YI	Y%	Y:A%	YpH	AW	AH	A%	HU	ApH
With CLA	22.04	45.66B	18.81	0.41A	32.34	54.75	5.74	40.42	5.23A	59.28	65.68A	9.03B
No CLA	21.65	46.11A	18.46	0.40B	31.81	53.45	5.75	40.78	4.86B	59.73	61.70B	9.10A
3 days	21.71	44.80C	19.90A	0.44A	31.53B	52.62B	5.72B	41.50A	5.61A	60.15A	69.25A	8.87C
6 days	21.96	46.08B	18.86B	0.41B	31.99A	53.64B	5.75A	41.12A	5.49A	59.85A	68.53A	9.12B
9 days	21.87	46.78A	17.16C	0.37C	32.70A	56.03A	5.78A	39.18B	4.01B	58.51B	53.30B	9.20A
						P-value						
CLA	0.0949	0.0134	0.0829	0.0151	0.0839	0.1075	0.4336	0.4267	0.0185	0.1675	0.0227	0.0051
Storage	0.6662	<0.0001	<0.0001	<0.0001	0.0080	0.0021	0.0001	<0.0001	<0.0001	0.0001	<0.0001	<00001
CLA × storage time	0.0968	0.0382	0.5045	0.9789	0.3869	0.2862	0.9022	0.4210	0.4349	0.2351	0.4569	0.0087
CV (%)	7.18	4.15	9.44	11.07	6.57	10.23	0.91	7.73	25.37	3.85	21.66	2.05

Interactions

Yolk diameter (YD)

Dietary treatment	Storage time (days)			*P*-value	CV (%)
	3	6	9		
With CLA	44.48c	45.63bB	46.87a	<0.0001	0.13
No CLA	45.12b	46.52aA	46.68a	<0.0001	0.13

Albumen pH (ApH)

Dietary treatment	Storage time (days)			*P*-value	CV (%)
	3	6	9		
With CLA	8.86c	9.03bB	9.19a	<0.0001	0.02
No CLA	8.88b	9.21aA	9.20a	<0.0001	0.02

Statistically significant differences by Tukey's test (at $P = 0.05$) in the same column are indicated by different uppercase letters. Statistically significant differences by Tukey's test (at $P = 0.05$) in the same row are indicated by different lowercase letters.

percentage (A%) in the eggs from breeders fed CLA was not affected, but decreased during storage, which may reflect the results found for AW and AH.

The average HU found in this study was lower than expected for fertile eggs (>80, according to Macari *et al.* 2013), which can be explained by the age of the breeders used in the experiment (between 58 and 61 weeks), since older breeders usually show lower albumen height and poor internal egg quality (Oliveira and Oliveira 2013). In contrast, the dietary CLA resulted in higher HU values than in the control (65.68 vs 61.70), a positive effect indicating that CLA was able to maintain the internal quality of the eggs up to 6 days. As expected, HU values were reduced as storage time increased, especially at Day 9.

Table 2 also shows that the albumen pH was negatively affected by the storage time in the egg room, since higher values were found at 9 days of storage. It is well known that ApH naturally increases after oviposition, as a result of carbon dioxide loss through the eggshell and plays an important role in the embryo development; lower ApH may negatively affect hatchability (Decuypere *et al.* 2001). Also, the addition of CLA to the breeder diet resulted in lower ApH values and preserved internal quality of eggs stored for 6 days.

Yolk lipid content and oxidation

The dietary supplementation of breeder diet with CLA showed no effect on the content of total lipids (TL) in yolks; however, it was significantly reduced as the storage time increased (Table 3). This is not unusual in stored eggs and can be explained by the increase in osmotic pressure gradient between albumen and yolk, resulting in movement of water from the albumen to the yolk and dilution of the yolk components, as a consequence of the liquefaction process (Macari *et al.* 2013). In addition, this process can ultimately cause damage to embryo viability, as described previously (Deeming 2002), since lipids from the yolk are their main source of energy during development.

Although the storage time in the egg room increased the lipid oxidation in the yolk up to Day 6 (Table 3), TBARS values decreased on Day 9 of storage. This may suggest that secondary compounds of lipid oxidation (malonaldehydes, MDA) reacted with other components of the yolk, such as

Table 3. Averages of total lipid content (TL, %), secondary lipid oxidation compounds (TBARS, μmol MDA/kg yolk) and acidity index (AI, mg NaOH/g of lipid) in yolks, and significance of main effects and interactions.

Factor	TL	TBARS	AI
With CLA	30.71	0.41B	1.90
No CLA	30.61	0.51A	1.90
3 days	33.17A	0.42B	1.84
6 days	29.97B	0.76A	1.98
9 days	28.84C	0.19C	1.83
		P-value	
CLA	0.7937	0.0082	0.9950
Storage	<0.0001	<0.0001	0.1276
CLA × Storage	0.5527	0.0129	0.0053
CV	6.51	58.71	15.96

Interactions

TBARS

Dietary treatment	Storage time (days)			*P*-value	CV (%)
	3	6	9		
With CLA	0.29bB	0.76a	0.17b	<0.0001	0.02
No CLA	0.55bA	0.76a	0.21c	<0.0001	0.02

Acidity index (AI)

Dietary treatment	Storage time (days)			*P*-value	CV (%)
	3	6	9		
With CLA	1.90	1.83B	1.93	<0.0001	0.05
No CLA	1.79b	2.14aA	1.73b	<0.0001	0.05

Statistically significant differences by Tukey's test (at $P = 0.05$) in the same column are indicated by different uppercase letters; statistically significant differences by Tukey's test (at $P = 0.05$) in the same row are indicated by different lowercase letters.

amino acids, sugar and proteins, or even were transformed to MDA dimers or trimers, diminishing reactivity with thiobarbituric acid (Barriuso *et al.* 2013). This may also be associated with the reduced content of total lipids found in 9 days stored eggs.

At the same time, the dietary use of CLA in breeder diets seems to protect the yolk lipids from lipid oxidation, since TBARS values were lower than in the control (0.41 vs 0.51), which suggests an antioxidant effect of CLA, as previously described by Hayat *et al.* (2010). The CLA supplementation has been associated with an increase in superoxide dismutase and glutathione peroxide activity, neutralisation of free radicals and reduced damage to tissues and membranes, as well as formation of secondary products of lipid oxidation, such as malonaldehydes (Kim *et al.* 2005; Jiang *et al.* 2014). In our study, this antioxidant effect was clearly seen at the early stages of storage, particularly on Day 3 (Table 3), but faded as the storage time at the egg room increased.

The dietary CLA reduced acidity index (AI) in eggs after 6 days of storage, when compared with the control (not supplemented; Table 3); however, no differences were detected on Days 3 and 9 of storage and these results were not related to TBARS. Since AI values in eggs from CLA treatment remained unchanged throughout the storage, in contrast to control, it could indicate better preservation of lipids from rancidity.

Yolk fatty acid profile

The percentages of palmitic, stearic, oleic and linoleic acids were evaluated. In this study, the concentration of palmitic acid (C16:0) in the yolks was not affected by dietary CLA or storage time in the egg room (Table 4), as described previously by Keum *et al.* (2018). However, dietary CLA increased the percentage of stearic acid (C18:0), as reported by other authors (Shinn *et al.* 2015; Liu *et al.* 2017; Keum *et al.* 2018). Some authors have associated this increase in the fatty acid saturation of the egg yolks with the inhibition of the stearoyl-CoA desaturase enzyme (Δ9-desaturase) in the liver (Park *et al.* 2000); this enzyme is known for the desaturation of Δ9-*cis* into several unsaturated fatty acids (Cohen *et al.* 2002).

In contrast to Shinn *et al.* (2015), the dietary CLA did not affect oleic acid (C18:1) concentration in yolk. However, the concentration of oleic acid was reduced after 6 days of storage at the egg room, as reported by Cherian *et al.* (2007).

The proportion of linoleic acid (C18:2) in yolks was reduced by the dietary addition of CLA, and no effect of the storage time was observed. The results were similar to those found by Suksombat *et al.* (2006) and Shinn *et al.* (2015), that showed a significant decrease on the polyunsaturated fatty acids in the yolks from CLA dietary treatment.

Table 4. Fatty acid composition (g/100 g) of egg yolks, including palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids.

Factor	C16:0	C18:0	C18:1	C18:2
With CLA	28.34	9.40A	43.41	11.72B
No CLA	27.84	8.37B	42.63	13.72A
3 days	28.18	9.06	43.41A	12.66
6 days	28.18	8.95	42.34B	12.43
9 days	27.92	8.64	43.31A	13.07
		P-value		
CLA	0.3174	0.0019	0.0510	0.0004
Storage	0.8998	0.3946	0.0506	0.4975
CLA × Storage	0.8940	0.2547	0.2170	0.1667
CV	2.69	7.88	2.14	9.92

Statistically significant differences by Tukey's test (at $P = 0.05$) in the same column are indicated by different letters.

Although the concentration of the supplemented CLA in the breeder diet was low in this study, it modified the fatty acid composition of the yolks (Table 4), such as the increased of saturated fatty acids (C18:0) and decreased polyunsaturated fatty acids (C18:2), as reported previously by Liu *et al.* (2017) and Keum *et al.* (2018). This may be related to the lower progression of lipid oxidation in yolks found in TBARS and AI analysis (Table 3), and may also reduce embryo survival (Muma *et al.* 2006; Aydin and Cook 2009).

Conclusions

The dietary supplementation of the broiler breeder diet with CLA minimised the negative effects in the internal quality of the yolk and albumen and preserved the lipids from oxidation at the early stages of storage; however, it modified the fatty acid composition of the yolks.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare no conflicts of interest.

Declaration of funding. This research did not receive any specific funding.

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