Original Research Article

Calcium supplementation in low nutrient density diet for meat ducks improves breast meat tenderness associated with myocyte apoptosis and proteolytic changes

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ABSTRACT

To define the relationship between dietary nutrient density, calcium (Ca), and meat quality in meat ducks. A total of 288 male Cherry Valley SM3 medium ducklings were fed a common standard starter diet until d 14. At 15 d of age, ducks were randomly divided into 2 treatment groups and fed either a conventional diet or a low nutrient density (LND) diet. Compared with the conventional diet, the energy was reduced in the LND diet by 8.6% and 16.8% in grower (15 to 35 d) and finisher (36 to 56 d) phases, respectively, while other essential nutrients were kept proportionate to energy. The LND diet decreased the shear force ($P < 0.05$) and increased the lightness values of the pectoralis muscle when compared to the conventional diet, suggesting that LND diet exerted a beneficial role in meat quality. Subsequently, the effects of grated Ca in the LND diet on meat quality of pectoralis muscle were evaluated. A total of 576 male ducklings were fed a common starter diet until d 14, followed by feeding 4 LND diets with 0.5%, 0.7%, 0.9%, and 1.1% Ca. The results show that LND diets with 0.7% or more Ca decreased the shear force of pectoralis major muscle in 42-d-old meat ducks ($P < 0.05$). To explore the mechanism underlying Ca and tenderness, data from birds fed either 0.5% or 1.1% Ca in the LND diet indicated that birds fed 1.1% Ca exhibited lower shear force, upregulated *calpains 1* expression, and higher calpains activity compared to those fed the LND diet with 0.5% Ca ($P < 0.05$). Moreover, the 1.1% Ca LND diet induced a higher myocyte apoptosis ($P = 0.06$) and upregulated mRNA expression of *caspase-3* ($P = 0.07$) in breast muscle. Our data suggest that LND diets with 0.9% or 1.1% Ca had a positive role in the tenderness of breast meat, particularly the enhancing effect of 1.1% Ca LND diet on tenderness seems to be associated with proteolytic changes of myofibrillar proteins and myocyte apoptosis in meat ducks.

1. Introduction

The consumption of duck meat has increased continuously over the past decades due to important improvements in meat production and the unique taste and nutritional characteristics of duck meat, e.g., flavor, aroma, high composition of essential amino acids and high percentage of polyunsaturated fatty acids (Olayiwola, 2006). However, the intensive genetic selection and/or use of high nutrient dense diets in poultry in order to obtain heavier body weight (BW) and higher muscle yield has introduced some changes in the meat quality traits such as pH, color, water-holding capacity (WHC) and tenderness (Huang and Ahn, 2018; Kokoszyński et al., 2019; Le Bihan-Duval et al., 2008; Witkiewicz et al., 2004). These quality traits are critical to consumers' initial selection of poultry meat as well as for final product satisfaction. Particularly in meat ducks, compared with non-selected ducks, the

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pectoralis major muscle was characterized by smaller per cent of red fibers and higher per cent of white fibers (Witkiewicz et al., 2004). Similarly, studies with broilers indicated that a dramatic improvement in growth rate and muscle size negatively affected meat quality, as reflected by an increased incidence of meat quality defects such as spaghetti meat, white stripping, woody breast, and pale-soft-exudative meat as a result of adversely modifying the structure, metabolism and repair mechanisms of muscles (MacRae et al., 2006; Maiorano, 2017; Velleman, 2015; Velleman et al., 2014).

The most important nutritional factor that influences the growth rate and economic benefit of meat poultry is dietary nutrient density since it determines growth performance, carcass quality (Zhao et al., 2009), and meat quality (Wang et al., 2013). Increase of the dietary nutrient density resulted in higher BW, meat yield, and better feed conversion ratio (Nahashon et al., 2005; Wang et al., 2013), whereas a high nutrient density diet was also found to lead to higher abdominal fat (Nahashon et al., 2005) and nitrogen excretion (Bregendahl et al., 2002). Furthermore, dietary nutrient density is an essential factor in meat quality. Indeed, it was reported that increasing dietary nutrient density significantly decreased meat pH and oxidative stability of thigh meat compared with low nutrient density (LND) diets in 42-d-old broilers (Mirshekar et al., 2013). Previous published results demonstrate that a high nutrient density diet was associated with notably larger myofiber area and lower fiber density, higher shear force, as well as higher moisture and protein content in broiler meat (Wang et al., 2013). There is evidence showing that feeding a high nutrient density diet decreased cooking loss and drip loss percentage, but apparently did not change the color of breast muscle in meat ducks (Gheisar et al., 2015). However, some other studies that focused on the effects of dietary protein levels on meat quality found that breast meat from the 13.5% crude protein (CP) group had a higher pH with a lower drop loss and shear force and a higher yellowness when compared with those from 17.5% CP group in Pekin ducks (Wang et al., 2020). These findings indicate that the effect of dietary nutrient density on quality characteristics of meat-type birds is poorly understood.

In addition, some minerals such as calcium (Ca) have been receiving more attention with regard to meat quality, especially in relation to tenderness, even though the main ability of Ca is to maintain serum Ca homeostasis and bone remodeling to contribute to bone mineralization and prevent skeletal deformations (Theobald, 2005). For example, we recently found that an LND diet with suitable dietary Ca level could improve bone quality of tibia and sternum by decreasing BW and suppressing bone resorption (Zhang et al., 2018, 2019). Data from practical experiences and scientific evidences have shown that infusing a calcium chloride (CaCl$_2$) solution into whole carcasses or cuts of meat enhances tenderness (Jaturasitha et al., 2004; Lawrence et al., 2003). Increasing plasma Ca by dietary 25-hydroxycholecalciferol was associated with a potential improvement in beef tenderness (Carnagey et al., 2008). The proposed mechanism to improve tenderness by Ca is probably via disruption of the myofibrillar network, altering the protein-to-protein interaction, and, more important, modifying the calpain protease system (Koohmaraie et al., 1989; Morgan et al., 1991; Wu and Smith, 1987). It is well established that the calpain protease system consists of µ-calpain, m-calpain and the endogenous inhibitor calpastatin (Goll et al., 2003) and is mainly responsible for postmortem proteolysis and subsequent tenderization of meat (Hwang and Thompson, 2001). The relationship between Ca and calpain enzyme has been confirmed in previous studies. For instance, CaCl$_2$ infusion increased the activity of calpain enzymes and increased tenderness of lamb meat (Koohmaraie et al., 1989). Injecting CaCl$_2$ was also found to improve beef tenderness by activating calpain earlier postmortem (Colle et al., 2018). Moreover, apoptosis is considered to be correlated with postmortem tenderization (Chen et al., 2011; Zhang et al., 2013). As a key enzyme in cell apoptosis, caspase-3 can cause myofibril fragmentation during postmortem tenderization (Kemp and Parr, 2008). In support of this, existing data suggested that muscle tenderization would be promoted with higher caspase-3 activity when treated with Ca^{2+} (Chen et al., 2011). However, the concept of the combination of LND diet with supplemental Ca has so far, to the best knowledge of the authors, not been addressed in meat ducks.

As noted above, we hypothesized that using an LND diet with appropriate Ca is probably beneficial to the meat quality in meat ducks. Specifically, the objectives of this work were as follows: 1) to study the effects of dietary nutrient density on meat quality of meat duck at different ages, and 2) to understand the relationship between Ca level in LND diets and meat quality, especially tenderness. The mechanism underlying the role of Ca in the meat tenderization process was also evaluated. These results, therefore, will bring a promising strategy for promoting duck meat production.

2. Materials and methods

2.1. Animal care and experimental design

Two experiments were conducted at the Sichuan Agricultural University Poultry Research Farm, and all procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (approval No. SAU-19-121). In these experiments, 1-d-old male Cherry Valley SM3 medium meat ducks, obtained from a local hatchery, were reared in an electrically heated and thermostatically controlled confinement building on floor cages (length × width × height, 2.2 m × 1.2 m × 0.9 m), and were allowed ad libitum access to diet and tap water containing no detectable Ca. The temperature in the room was 32 °C for 1 to 7 d and then the temperature was linearly lowered to 22 °C by 20 d. The light schedule was 23 h of light and 1 h of dark throughout the experimental period. Relative humidity and air movement were adjusted to bird age, following normal management practices. Birds were fed a starter (1 to 14 d), grower (15 to 35 d) and finisher (36 to 56 d) diet as pellet.

2.1.1. Experiment 1

In order to evaluate the impact of dietary nutrient level (conventional diet vs. LND diet) on the meat quality of meat ducks, 288 male ducklings were allocated to 2 treatment groups with 8 replicate pens (18 birds/pen). All birds were fed the same starter diet till 14 d. At 15 d of age, birds were given the LND diets or the conventional diets that were formulated according to the China Agricultural Industry Standards (NY/T, 2122-2012) recommendations. Specifically, compared with the conventional diets, the apparent metabolizable energy (AME) in the LND diets was reduced to 11.10 and 10.27 MJ/kg for grower and finisher diets, respectively, based on our previous studies (Zhang et al., 2019), whilst the ratio of CP, digestible essential amino acids, and other nutrients to AME was maintained (Table S1). Feed intake by pen was recorded during the trial period. At 35, 42, 49 and 56 d, after fasting for 12 h, one bird in each pen was selected based on the average BW of the pen. Selected birds were euthanized by cervical dislocation and the breast muscle (pectoralis major muscle plus pectoralis minor muscle) was removed to evaluate breast yield. Pectoralis major muscle was used for meat quality determinations.

2.1.2. Experiment 2

The objective of this experiment was to evaluate the effect of Ca level in LND diets on meat quality of meat ducks, which were reared for 42 d. A total of 576 ducklings were fed the same starter diet for 14 d, and subsequently divided into 4 treatments with 8 replicates of 18 birds. The treatments were Ca at 0.5%, 0.7%, 0.9% and 1.1%. The LND diets in this trial were formulated as in Exp. 1 except for Ca and phosphorus (P) as shown in Table S2. Feed intake by pen was recorded during the trial period.

Upon completion of the feeding experiment, feed was withdrawn for 12 h and 2 birds in per pen were selected based on the average BW of the pen. Ducks were euthanized by cervical dislocation and the breast muscle (pectoralis major muscle plus pectoralis minor muscle) was removed. The first duck was used to evaluate breast yield and meat quality. For the second bird, in addition to the determination of the meat quality, a part of pectoralis major muscle from birds fed LND diets with either 0.5% or 1.1% Ca was obtained and stored at −80 °C until required.

2.2. Analyses of Ca, P, AME and CP in diets

Ca and P contents of feeds were determined through ethylene diamine tetraacetic acid titration (EDTA) and ammonium metavanadate colorimetry, respectively, and values were presented on the basis of dry matter (DM) weight. For AME and CP determination, one 35-d-old bird per pen was randomly selected (8 ducks per treatment, 40 ducks in total) and transferred to metabolic cages (1 duck per cage) and fed with the corresponding trial diets mixed with 0.3% chromic oxide (Cr_2O_3). After a 3-d adaptation period, excreta from each cage were collected daily for the next 72 h. After each collection of feces, 10% hydrochloric acid was added to fix excreta nitrogen. Before chemical analysis, the fecal samples were dried at 60 °C for 72 h, after which they were finely ground to a size that could pass through a 1-mm screen. Then, all the feed and fecal samples were analyzed for DM, Cr, CP, and AME as previously described (Zeng et al., 2015).

2.3. Breast yield

Birds were sacrificed by cutting the neck and bled for 5 min. Subsequently, the bird was defeathered after being submerged in water at 60 °C for 2 min, and the carcass weight was calculated by subtracting the weight of feathers and blood from the live weight. Then beast muscle including pectoralis major muscle and pectoralis minor muscle was removed from the carcass and trimmed of adipose tissue. The breast muscle was weighed on electronic balance (WANT Balance Instrument Co., Ltd, Jiangsu, China), accurate to 0.1 g. The breast yield expressed as a percentage of carcass weight was calculated.

2.4. Meat quality measurement

Pectoralis major muscle was used for meat quality assessments including color, pH, drip loss, and tenderness. The color as L* (lightness), a* (redness) and b* (yellowness) values were measured using a Minolta Chromameter CR-300 (Minolta, Japan) with a measuring area of 50 mm diameter. The chroma meter was calibrated against white reference tile (L* = 100.00, a* = 0.32, b* = 0.33). Each object was measured 3 times to minimize the inter-observer variability. pH of the meat was determined at 45 min postmortem using an automated pH probe (pH-STAR, SFK-Technology, Denmark). The average pH value was based on 3 recordings on the same muscle area. WHC was measured via drip loss

by the method described by Franco et al. (2011). Tenderness was measured through the Warner-Bratzler shear force using Texture Analyzer (TA. XT. plus. Stable Micro systems, UK) following Tang et al. (2008).

2.5. Detection of apoptotic nuclei

The changes of apoptotic nuclei were recognized by terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) as described (Cao et al., 2010). Muscle tissues were cut into 5-μm-thick sections and blocked in 3% H_2O_2 and 100% methanol to wipe off the endogenous peroxidases. Subsequently, they were washed with 1× phosphate buffer saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $ClNa_2^+$, 1.4 mmol/L KH_2PO_4, pH 7.4). The sections were blocked with goat antiserum for 30 min and washed with 1× PBS for 30 min, followed by reaction with TUNEL reaction mix (1:9) for 60 min at 37 °C in the dark. The positive control was incubated with 5.1 unit/mL DNase I before adding TUNEL reaction mix. Negative control was incubated with label solution without terminal transferase instead of TUNEL reaction mix. The micrographs of the sections were taken and analyzed using a fluorescence microscope (CKX31, Olympus, Japan) at a magnification of 200× . The apoptosis rate was expressed as the number of positive nuclei per whole muscle section.

2.6. Determination of calpains and calpastatin activity

The protocol was conducted as reported previously (Biswas et al., 2016). Briefly, about 3 g of finely cut samples were homogenized with 6 mL extraction buffer (10 mmol/L EDTA, 0.05% 2-mercaptoethanol [MCE], and 20 mmol/L tris-base, pH 5.9). The sample extracts obtained were purified using dialysis tube of 12 kDa MWCO cellulose filters (Sigma−Aldrich) with dialysis buffer containing 40 mmol/L tris-base, 5 mmol/L EDTA, and MCE (1:20) for overnight at 4 °C. Anion exchanges column chromatography with various NaCl concentrations was performed to separate μ-calpains, m-calpains, and calpastatin.

Then, aliquots of 0.5-mL pooled fractions containing μ- or m-calpains were allowed to react with 1.5 mL of assay buffer (100 mmol/L tris-base, 5 mmol/L $CaCl_2$, 1 mmol/L NaN_3, 5 mg/mL casein and 10 mmol/L MCE) for 60 min at 25 °C, followed by the reaction was terminated by adding 5% trichloroacetic acid (TCA). The soluble proteins of each fraction were precipitated and measured at A_{278} (Thermo Scientific, China). Particularly, $CaCl_2$ in the reaction mixture was replaced with 10 mmol/L EDTA for determining Ca^{2+} independent proteolytic activity of each fraction, and thus the Ca^{2+} dependent proteolytic activity was obtained by absorbance at A_{278} in the $CaCl_2$ reactions subtracted that of the presence of EDTA. Therefore, total activity was calculated by multiplying Ca^{2+}-dependent proteolytic activity by the dilution factor and defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at A_{278} after 60 min at 25 °C. In addition, calpastatin activity defined as inhibiting one unit of m-calpain was measured by incubating appropriate amounts of pooled fractions containing calpastatin and m-calpain.

2.7. RNA isolation and RT-PCR

RNA from breast muscle was extracted, and then reverse transcribed into cDNA using a reverse transcript kit (Takara, Japan). Beta ryanodine receptors (RYR2), Ca^{2+}-storage protein calsequestrin (CASQ2), μ-calpains (Capn1), m-calpains (Capn2), calpastatin (Cast), and Caspase 3 mRNA were quantified by RT-PCR using a 7900 Fast

Real-Time PCR System (Applied Biosystems, USA). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin*. Primer sequences can be found in Table S3.

2.8. Statistical analysis

The statistical analysis was performed using SAS statistical software (version 9.2, SAS Institute, Cary, NC). All data were presented as means ± standard deviation. Statistical significance was assigned at $P < 0.05$. Data between the conventional and LND diet were analyzed by two-way analysis of variance (ANOVA) with Tukey's post hoc comparison. The model included dietary nutrient density, age and their interaction, as in the following model: $y_{ijl} = \mu + a_i + b_j + (ab)_{ij} + e_{ijl}$, where y_{ijl} is value of the studied feature; μ is population mean; a_i is effect of the i diet (i = the conventional or LND diets); b_j is j effect of the j age; $(ab)_{ij}$ is diet and age interaction effect; e_{ijl} is random effect.

The effect of Ca in LND diet was tested by one-way ANOVA and Tukey's post hoc test were used, with the calculations being performed using the following model: $y_{il} = \mu + a_i + e_{il}$, where y_{il} is value of the studied feature; μ is population mean; a_i is effect of the i Ca concentration in LND diet (i = 0.5%, 0.7%, 0.9% or 1.1%); e_{il} is random effect.

A linear model was applied to determine the effects of different age or different dose levels (0.5%, 0.7%, 0.9% or 1.1%) of Ca in LND diet on meat characteristics with the following model: $y_{il} = \mu + a_i + e_{il}$, where y_{il} is the value of the analyzed trait, μ is the overall mean of the analyzed trait, a_i is the effect of duck age or Ca level in LND, and e_{il} is random error.

In addition, a quadratic contrast was also used to determine the effects for different dose levels (0.5%, 0.7%, 0.9% or 1.1%) of Ca in LND diet in Exp. 2. Differences between 0.5% and 1.1% Ca groups were evaluated using a two-tailed unpaired *t*-test or the Mann−Whitney U test for normally or non-normally distributed datasets, respectively.

3. Results

3.1. Diets analysis

Nutrient composition data are presented in Table 1. In Exp. 1, the AME of the conventional diets and LND diets were 12.23 and 11.25 MJ/kg for grower diets, and 12.38 and 10.21 MJ/kg for finisher diets, respectively. These diets had a constant AME:CP ratio across conventional diet and LND diet, which corresponded to 0.70 vs. 0.71 and 0.77 vs. 0.77, for grower and finisher diets, respectively. In

Exp. 2, the analyzed concentration of Ca in LND diets was close to the intended 0.5%, 0.7%, 0.9% and 1.1% for the grower-finisher diet. These data confirmed proper preparation of experimental diets in this study.

3.2. Breast yield response to dietary nutrient density and age

As illustrated in Fig. 1, age remarkably impacted BW and breast yield, and both significantly increased with age ($P < 0.01$). The LND diet led to lower BW and breast yield ($P < 0.001$), but it had little effect on feed intake (Fig. S1A). There were no interactions between diet and age for breast yield ($P > 0.05$).

3.3. Meat quality responses to dietary nutrient density and age

The dietary nutrient density and the interactions of nutrient density and age did not significantly change the pH$_{45min}$, drip loss, a*, and b* of breast muscles ($P > 0.05$), but feeding the LND diet resulted in lower shear force at 49 d and higher L* at 42 d ($P < 0.05$, Fig. 2). The effects of age on L* and a* and shear force were significant (Fig. 2), with a lower L* and pH$_{45min}$, as well as a higher a* and shear force of breast muscle as the birds matured ($P < 0.05$; Fig. 3).

3.4. Breast yield and meat quality response to LND diets with various Ca

Combining the association of Ca and meat quality and the positive role of the LND diet prompted us to evaluate the effects of Ca supplementation in the LND diet on meat quality of 42-d-old ducks. No differences were found in BW and breast yield (Fig. 4), feed intake (Fig. S1B), and meat quality of breast among Ca administration levels in the LND diet, with the exception of shear force. The LND diets with 0.7% or more Ca resulted in linear and quadratic lower shear force as compared with the 0.5% Ca LND diet ($P < 0.01$; Fig. 5).

3.5. LND diet with 1.1% Ca supplementation improves tenderness of breast muscle

Meat quality assessment of breast muscle using the second bird indicated that birds fed the LND diets with the 0.5% or 1.1% Ca for 42 d displayed no difference in meat color, pH$_{45min}$, and drip loss (Figs. S2A−C). When compared with 0.5% Ca LND diet, 1.1% Ca supplementation notably decreased the shear force of breast muscle in LND diets (Fig. S2D).

Table 1
Analyzed dietary AME, CP, Ca and total P content.

Item	Diets	AME, MJ/kg	CP, g/100 g	AME:CP, MJ/10 g	Ca, g/100 g	Total P, g/100 g
Starter (1−14 d)	Starter diet	12.49 ± 0.33	20.11 ± 0.89	0.62 ± 0.02	0.93 ± 0.03	0.68 ± 0.06
Exp. 1						
Grower (15−35 d)	Conventional diet	12.23 ± 0.31	17.43 ± 1.33	0.70 ± 0.04	0.89 ± 0.03	0.70 ± 0.03
	LND diet	11.25 ± 0.18	15.88 ± 1.11	0.71 ± 0.05	0.90 ± 0.04	0.69 ± 0.08
Finisher (36−56 d)	Conventional diet	12.38 ± 0.42	16.11 ± 0.78	0.77 ± 0.05	0.84 ± 0.06	0.74 ± 0.05
	LND diet	10.21 ± 0.49	13.22 ± 2.00	0.77 ± 0.11	0.88 ± 0.10	0.75 ± 0.06
Exp. 2						
Grower (15−35 d)	0.5% Ca LND diet	11.23 ± 0.03	16.01 ± 0.67	0.70 ± 0.03	0.48 ± 0.04	0.68 ± 0.05
	0.7% Ca LND diet	11.13 ± 0.77	16.02 ± 0.82	0.69 ± 0.06	0.71 ± 0.04	0.72 ± 0.08
	0.9% Ca LND diet	11.25 ± 0.18	15.88 ± 1.11	0.71 ± 0.05	0.90 ± 0.04	0.69 ± 0.08
	1.1% Ca LND diet	11.13 ± 0.46	15.77 ± 0.27	0.71 ± 0.04	1.03 ± 0.12	0.70 ± 0.13
Finisher (36−56 d)	0.5% Ca LND diet	10.15 ± 0.54	13.17 ± 0.96	0.77 ± 0.08	0.52 ± 0.05	0.73 ± 0.07
	0.7% Ca LND diet	10.14 ± 0.97	13.14 ± 0.62	0.77 ± 0.07	0.71 ± 0.09	0.76 ± 0.05
	0.9% Ca LND diet	10.21 ± 0.49	13.22 ± 2.00	0.77 ± 0.11	0.88 ± 0.10	0.75 ± 0.06
	1.1% Ca LND diet	10.12 ± 0.66	13.11 ± 2.12	0.77 ± 0.10	1.11 ± 0.16	0.74 ± 0.08

AME = apparent metabolizable energy; CP = crude protein; Ca = calcium; P = phosphorus; LND = low nutrient density.
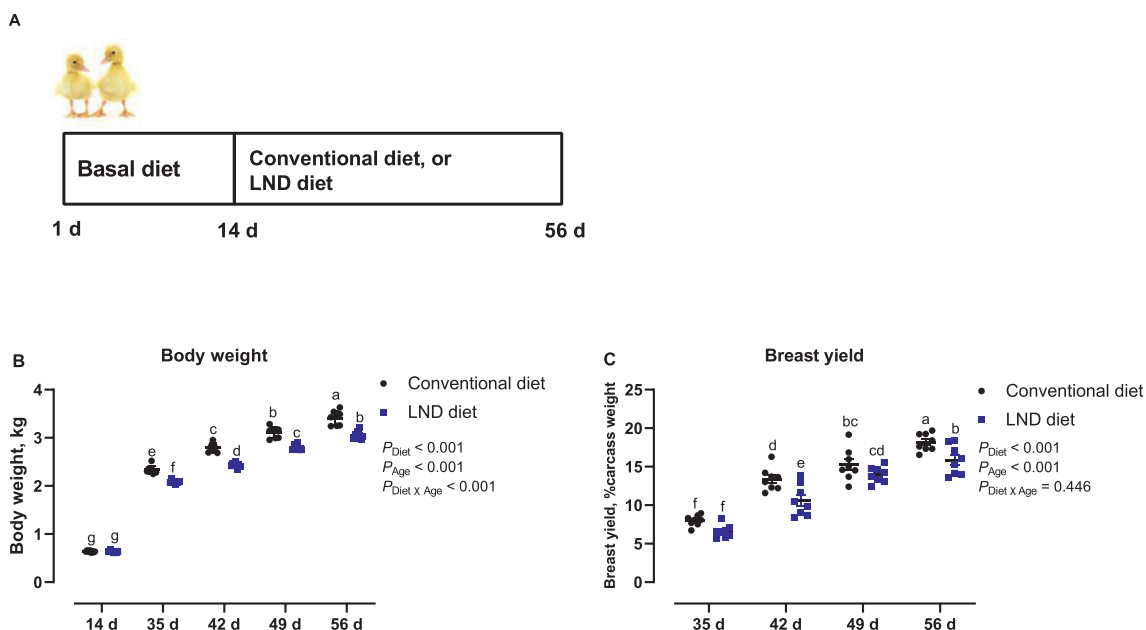
Fig. 1. Low nutrient density (LND) diet induces lower body weight and breast yield in meat ducks. (A) Schematic presentation of the experimental design. All birds were fed the same starter diet until 14 d, followed by the LND diet or the conventional diet until 56 d. (B) Body weight and (C) breast yield changes were assessed. Data represent means with standard deviation. Mean values with different letters are significantly different ($P < 0.05$).

### 3.6. LND diet with 1.1% Ca supplementation induces proteolysis and apoptosis during postmortem

Expression of *RYR2* and *CASQ2* both reflecting the Ca status, were determined, and showed that the mRNA level of *CASQ2* was elevated by the 1.1% Ca LND diet (Fig. 6A and B). In contrast, the

transcription of *RYR2* in breast muscle was not changed. Because calpastatin is the competitive inhibitor of μ- and m-calpains, the expression of genes encoding the calpain proteolytic system (*Capn1*, *Capn2*, *Cast*) were here reported as *Capn1*/*Cast* and *Capn2*/*Cast*. The *Capn1*/*Cast* mRNA ratio was higher in the 1.1% Ca group as compared to the 0.5% Ca group ($P < 0.05$), whereas the *Capn2*/*Cast*
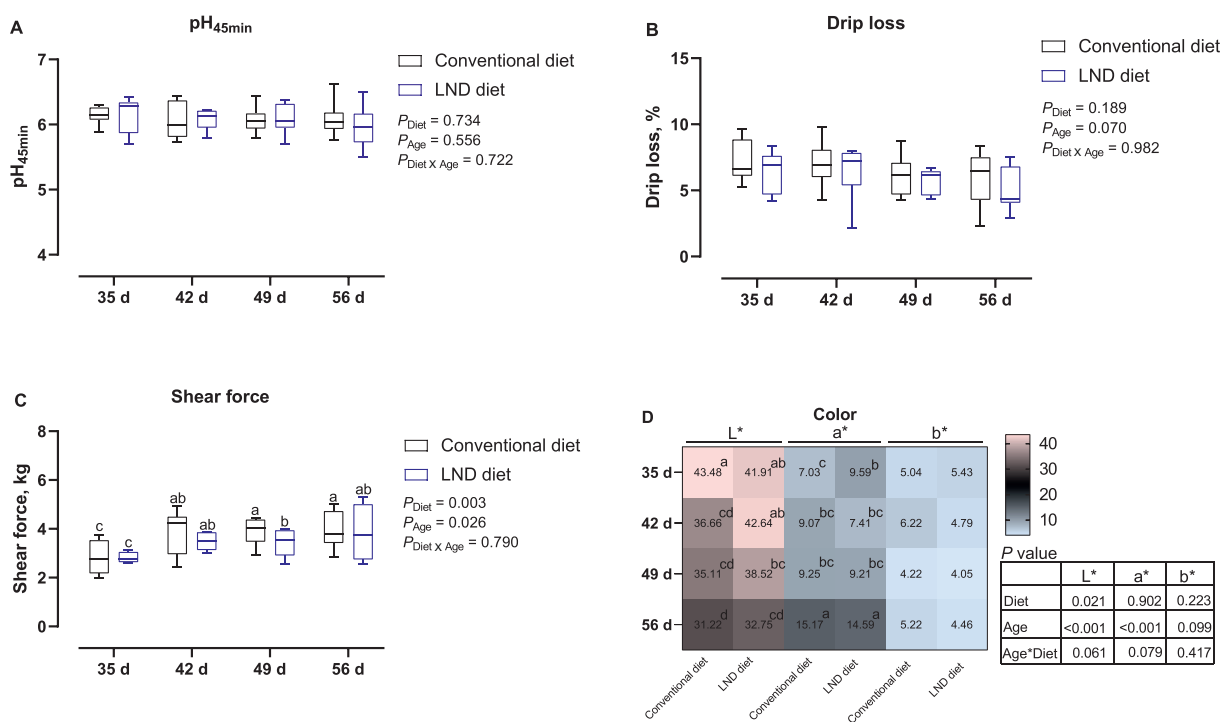
Fig. 2. Meat quality responses to dietary nutrient density and age. (A) pH$_{45min}$, (B) drip loss, (C) shear force, and (D) meat color. Mean values with different letters are significantly different ($P < 0.05$).
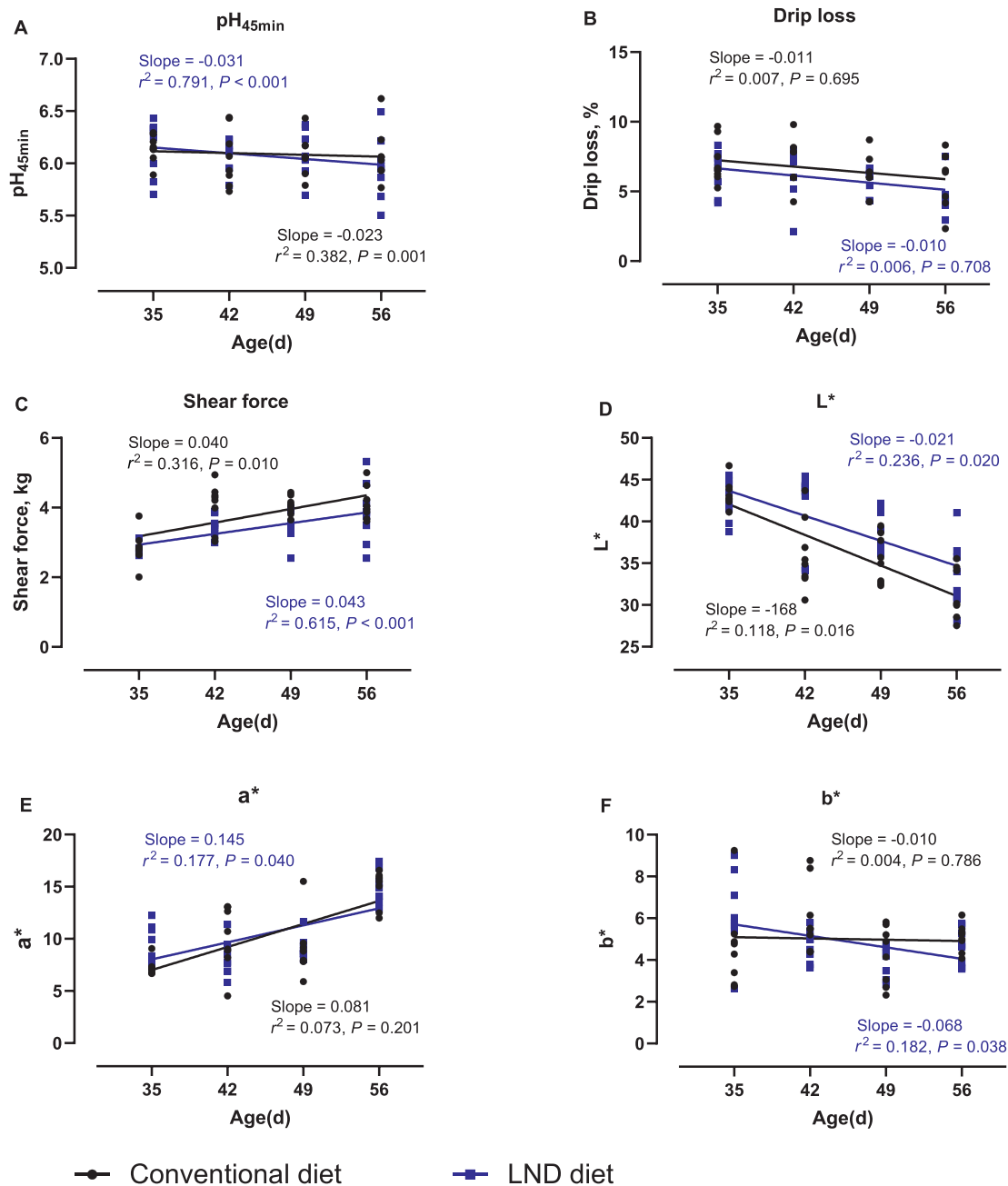
Fig. 3. The regression of (A) pH$_{45min}$, (B) drip loss, (C) shear force, (D) L* (lightness) value, (E) a* (redness) value, and (F) b* (yellowness) value of breast muscle came from birds given the conventional diets or the low nutrient density (LND) diets as a function of age.

ratio was similar between these experimental groups (Fig. 6C and D). Compared with the 0.5% Ca group, there was an increase in μ-calpains ($P < 0.05$) and m-calpains ($P = 0.063$), and calpastatin ($P = 0.097$) in the 1.1% Ca LND diets, indicating the activation of proteolysis.

Regarding apoptosis, the apoptotic nuclei counts demonstrated increased percentage of TUNEL positive nuclei in the LND diet with 1.1% Ca as compared to the 0.5% group, thus, birds fed 1.1% Ca exhibited more apotosis (Fig. 7A and B). The level of caspase-3 mRNA in the 0.5% Ca group was lower than that in the 1.1% Ca group ($P = 0.066$; Fig. 7C). These results indicate that the apoptotic process was obviously promoted by 1.1% Ca in the LND feed.

4. Discussion

Duck meat is a food of high nutritional quality, and consumer's interest in duck meat is growing. Previous studies in broilers have shown that an LND diet can enhance meat quality by improving oxidative stability and increasing pH of thigh muscle (Mirshekar et al., 2013). In addition, CaCl$_2$ injection has been found to provide enough Ca ions to activate calpain-2 early postmortem resulting in improved tenderness of beef (Colle et al., 2018). Thus, we hypothesized that using an LND diet with appropriate Ca may have favorable effects on the meat quality of ducks. In the current study, we provided evidence that
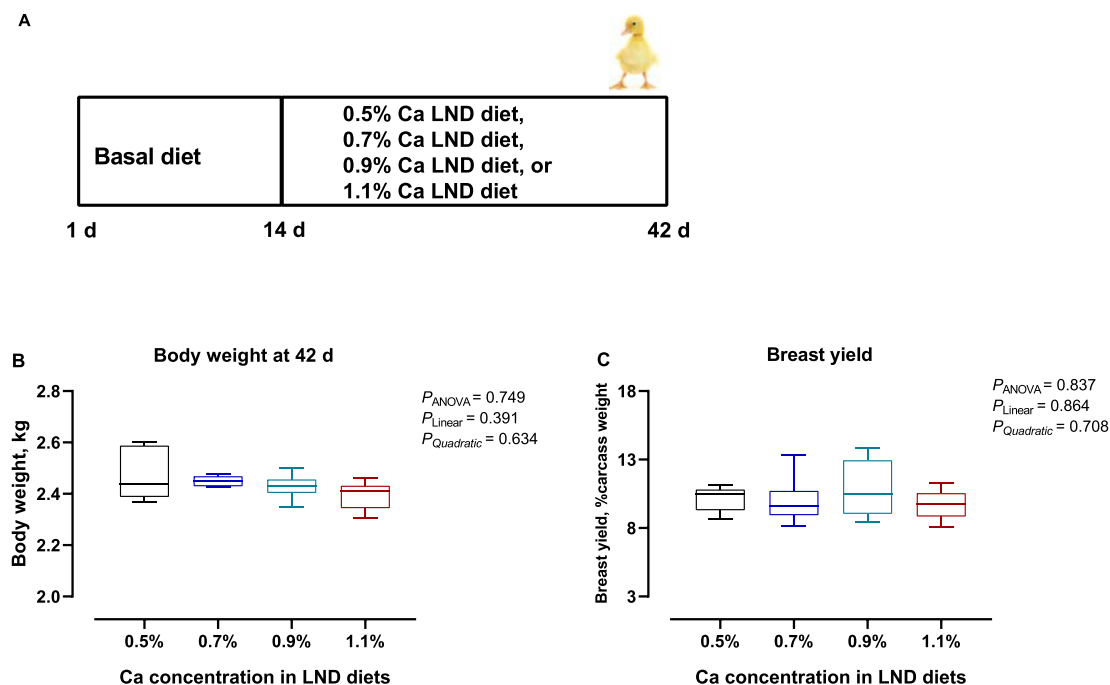
Fig. 4. Dietary Ca supplementation in low nutrient density (LND) diets did not change the body weight and meat yield in 42-d-old meat ducks. (A) Schematic presentation of experimental design in which 1-d-old ducks were fed a common starter diet till 14 d old, and subsequently fed LND diets with various Ca levels till 42 d old. (B) Body weight and (C) percentage of meat yield of carcasses was determined at 42 d.

reducing dietary nutrient density is beneficial to meat quality despite decreasing BW and breast yield. Furthermore, feeding LND diets with 0.9% or 1.1% Ca could improve meat quality through enhancing the tenderness of breast meat in 42-d-old meat ducks, and the positive role of the 1.1% Ca LND diet in tenderness was associated with proteolysis and myocyte apoptosis postmortem.

Previous studies indicated that a diet with high nutrient density resulted in heavier BW in growing broilers (Nahashon et al., 2005; Zhao et al., 2009). Similarly, in the present study, notably higher BW and breast yield were obtained by the conventional diets that included high AME and CP. However, some studies showed that breast yield was not influenced by a low compared with a high CP diet. Their explanation was that levels of essential amino acids, particularly lysine and methionine, were maintained in the low CP diets (Kamran et al., 2008). In this study, the essential amino acid contents were reduced accompanying the decreased CP concentrations in the LND diet. Sterling et al. (2006) reported that as dietary lysine decreased, breast yield was significant decreased. In addition, recent data showed that dietary nutrient density is also a key factor in meat quality (Meng et al., 2010; Mirshekar et al., 2013). Increasing dietary nutrient density was associated with a decrease in pH of thigh muscle (Mirshekar et al., 2013) and a darker color of the right loin in growing-finishing pigs (Meng et al., 2010). The nutrient density used in the present study had no effect on the pH and drip loss of breast muscle which is consistent with previous studies (Fanatico et al., 2007; Wang et al., 2013). It is well-established that WHC is influenced by tissue fat and water content. As the amount of fat increases in the tissue the moisture decreases, as a result, WHC increases. The fact that drip loss of breast muscle was comparable across treatments in the present study might thus be related to indistinctive ratios of intramuscular fat and water in breast

muscle (Peter et al., 1997). Besides, lower pH could prompt muscle fiber contraction, causing more drip loss (Tang et al., 2013), thus the indifferent pH might also explain why the drip loss of breast muscle was similar among treatment groups. Furthermore, we observed that the LND diet increased the lightness (L*) values and decreased the shear force of breast muscle, suggesting that LND diet exerted a beneficial role in meat quality. Of note, in the present experiment slaughter age had large effects on color and tenderness of the breast muscle. A greater force had to be applied to cut breast muscles in older birds than in younger ones, which is consistent with previous findings in broilers (Chen et al., 2007), geese (Uhlirova et al., 2018), and ducks (Kokoszyński et al., 2021; Muhlisin et al., 2013). This is probably associated with the lower muscle fiber diameter of lighter ducks. It was reported that the lighter ducks from genetic reserve flocks have a smaller diameter of white and red fibers and a higher percentage of red fibers than the heavier ducks in selected pedigrees (Witkiewicz et al., 2004). Taking meat color into account, the change in color indicated by a lower L*, and a higher a* of breast muscle as the birds matured could be related to the development of the muscle tissue, i.e., increases in cross-sectional area of muscle fiber or increases in collagen content and cross-linking with age (Dransfield and Sosnicki, 1999). In addition, the darker color of meat in older ducks probably resulted from a significantly higher content of haem pigments accompanied by better blood supply to the muscles (Kokoszyński et al., 2021).

From the comparison between conventional and LND diet it can be perceived that the reduction in dietary nutrient density favors the quality characteristics of the breast muscles in meat ducks. Based on evidence that illustrates the positive role of Ca in tenderness of beef (Carnagey et al., 2008; Jaturasitha et al., 2004; Lawrence et al., 2003), it was expected that the addition of
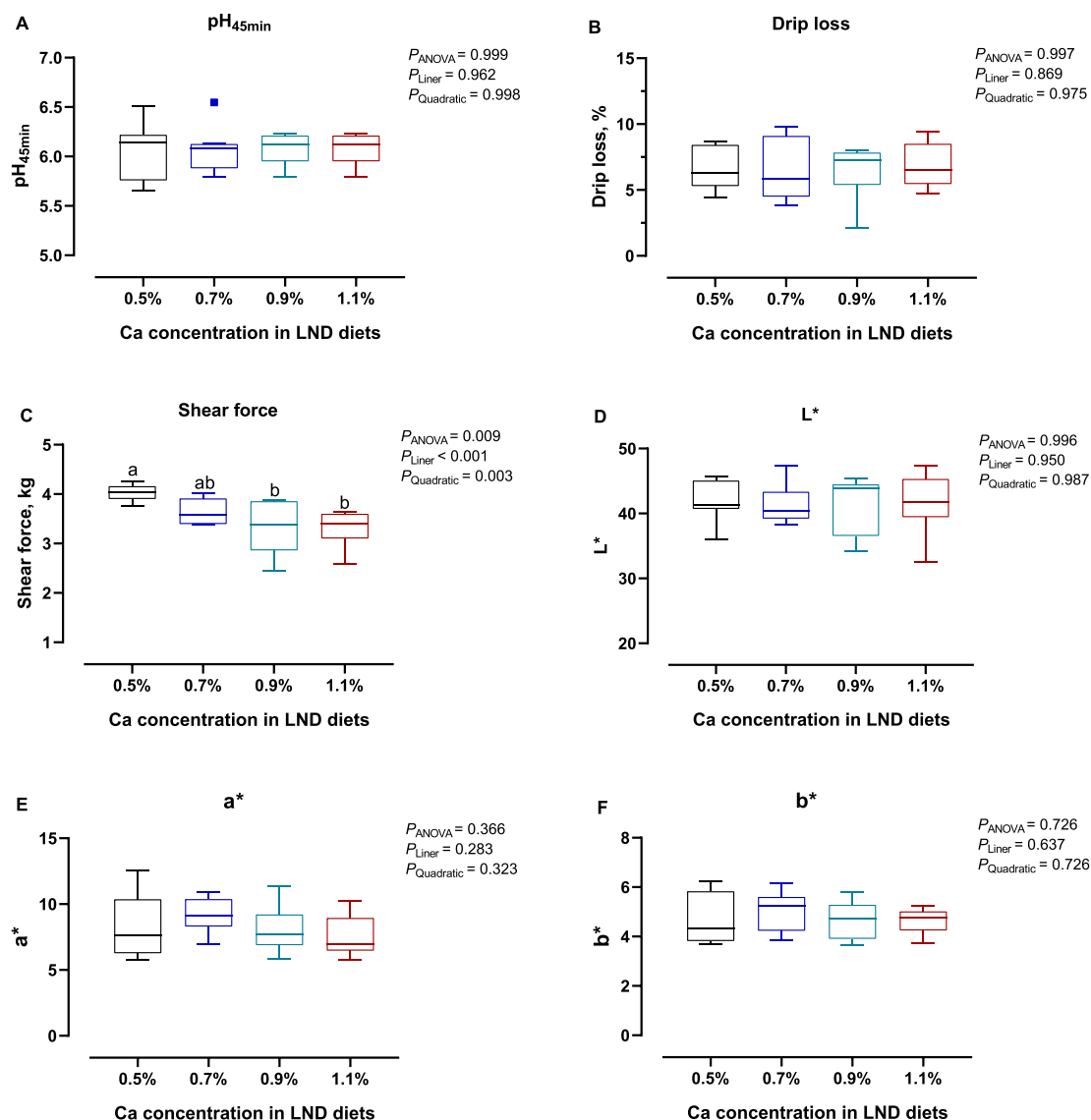
Fig. 5. The impact of Ca supplementation to the low nutrient density (LND) diets on (A) pH_{45min}, (B) drip loss, (C) shear force, (D) L* (lightness) value, (E) a* (redness) value, and (F) b* (yellowness) value of breast muscle. Mean values with different letters are significantly different ($P < 0.05$).

appropriate Ca to the LND diet could be even more benign to meat quality. In our study, Ca supplementation in the LND feed had no apparent influence on pH, drip loss, and color of breast muscles, whereas a significant positive effect of Ca in LND diet on the tenderness of meat was noticed for 42 d of age meat ducks. Analogously, a study that used $CaCl_2$ to regulate the postmortem tenderization process of duck breast muscle found that duck muscle tenderness could be significantly raised by soaking with $CaCl_2$ (He et al., 2019). On the contrary, no significant relationships were found between Ca and tenderness of meat from growing-finishing pigs (Shelton et al., 2004). The discrepancies regarding the effects of dietary Ca on tenderness in various studies could be explained by the species, the amount of collagen, intramuscular fat content, and the size and type of muscle fibers, among other factors.

The calpain system is considered to be correlated with postmortem tenderization (Hwang and Thompson, 2001), and duck muscle tenderness that was enhanced by $CaCl_2$ was associated with the activation of calpain system (He et al., 2019). It was reported that improvement of meat tenderness could be a result of activating calpain enzymes induced by increasing dietary Ca (Colle et al., 2018; Koohmaraie et al., 1989). Calpastatin is a calpain specific inhibitor (Koohmaraie and Geesink, 2006), therefore, the calpains/calpastatin ratio can be deemed to be related to beef tenderness. In the present study, high dietary Ca manipulation (1.1% vs. 0.5% Ca in LND diet) promoted *CASQ2* expression, a high capacity Ca^{2+}-binding protein that stores Ca^{2+} until it is needed again for muscle contraction (Rossi and Dirksen, 2006), suggesting that dietary Ca addition could promote Ca deposition in meat. It also indicated that the calpain system of duck could be well activated with the LND diet with 1.1% Ca treatment after death, evidenced by upregulated *Capn1/Cast* mRNA. In line with this, existing data suggested that $CaCl_2$ infusion increased the activity of calpain enzymes and increased tenderness of lamb
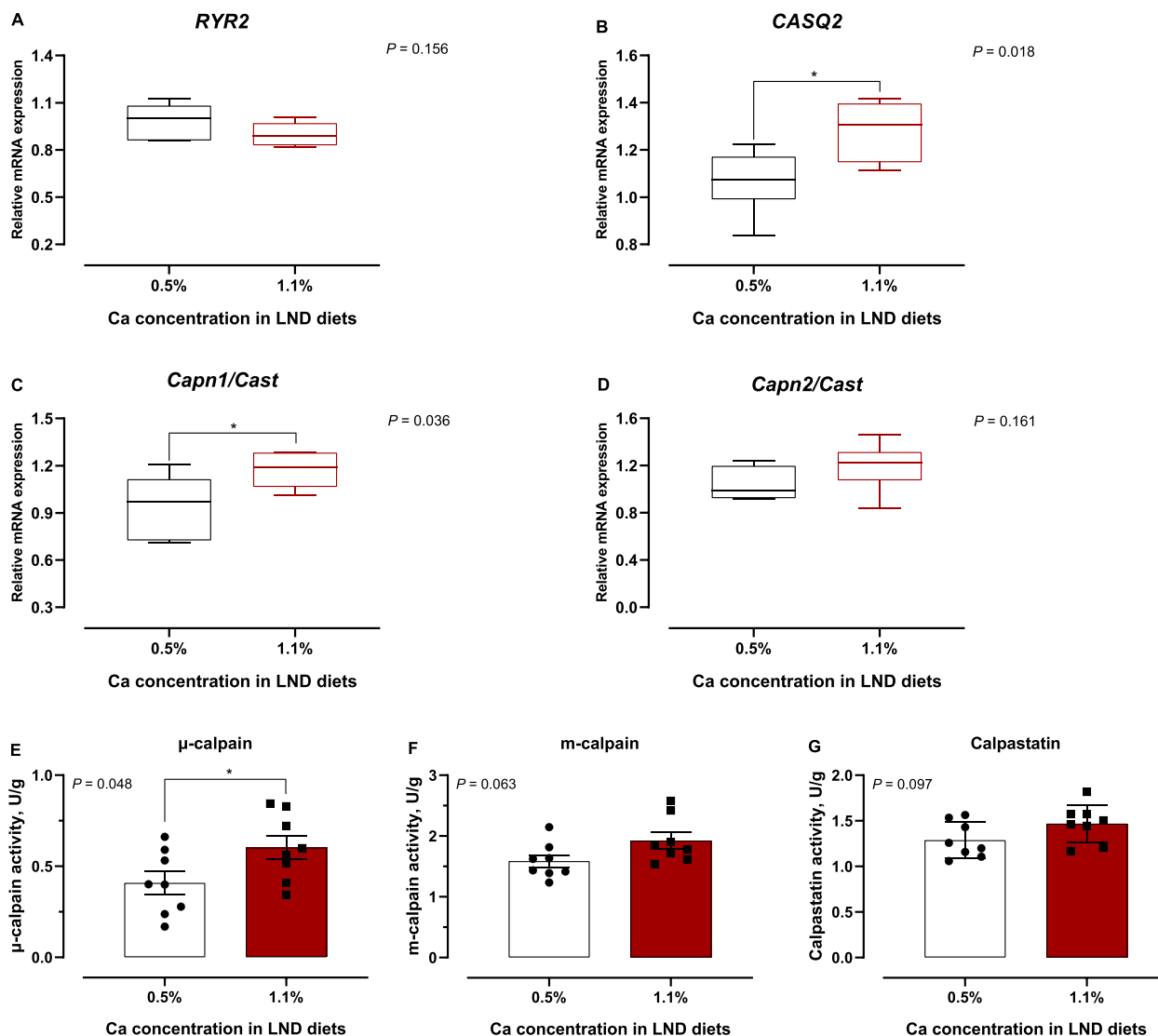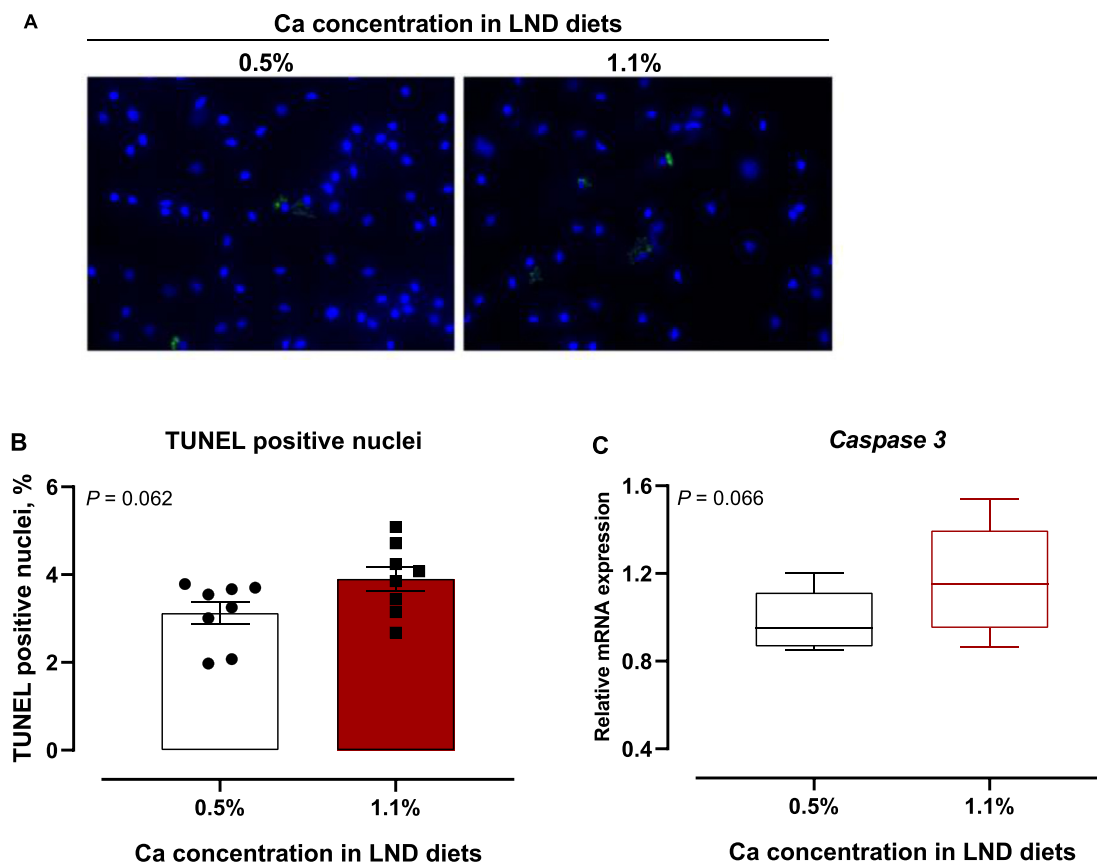
Fig. 6. Low nutrient density (LND) diet with 1.1% Ca activated the proteolysis of breast muscle. The Ca-regulating genes include (A) ryanodine receptors (*RYR2*) and (B) Ca^{2+}-storage protein calsequestrin (*CASQ2*), as well as the calpain protease system i.e., (C to D) μ-calpain (*Capn1*), m-calpain (*Capn2*) and calpastatin (*Cast*); all measured using RT-PCR. The activity of (E) μ-calpain, (F) m-calpain and (G) calpastatin were also determined using casein as a substrate. Data represent means with standard deviation. *$P < 0.05$.

meat (Koohmaraie et al., 1989). Injecting $CaCl_2$ was found to improve beef tenderness by activating calpain earlier postmortem (Colle et al., 2018). Moreover, apoptosis is considered another factor in postmortem tenderization (Chen et al., 2011; Zhang et al., 2013). Caspase-3 is a key enzyme in cell apoptosis, and it can cause myofibril fragmentation during postmortem tenderization (Kemp and Parr, 2008). The higher percentage of the apoptotic nuclei counts and elevated mRNA level of caspase-3 in birds fed the LND diet with 1.1% Ca might explain the improvement in tenderness of breast muscle. This corroborates with a previous study saying that muscle tenderization would be promoted with higher caspase-3 activity when treated with Ca^{2+} (Chen et al., 2011). However, another study pointed out the apoptosis-related enzymes, including caspase-3, Na^+/K^+-ATPase, and Ca^{2+}-ATPase, seemed to have no significant effects on duck muscle tenderness, and further it was speculated that these enzymes should be, at least, not the main factors in duck postmortem (He et al., 2019). Further studies are required to assess

the role of cell apoptosis in the actions of dietary Ca on meat quality.

In summary, a limitation of the study was that the insufficient sample size that were used to access the mechanism underling Ca action on meat quality. In the present study, only 2 Ca levels in LND diet were used, i.e., 0.5% and 1.1% Ca in LND diets. Increasing the tested concentration gradients would find more accurate threshold and more scientific conclusions. Thus, we admit the possibility that some of our conclusions may include overestimation or underestimation of roles regarding the LND diet with various Ca in enhancing tenderness of breast muscle in ducks. Collectively our data indicate that the lightness and tenderness of breast muscles exhibited apparent decreases with slaughter age. LND diets with 0.9% and 1.1% Ca were beneficial to improve the tenderness of breast meat from meat ducks, particularly the enhancing effect of 1.1% Ca LND diets on tenderness seems to be associated with proteolytic changes of myofibrillar proteins and myocyte apoptosis during postmortem of duck meat.

A

Ca concentration in LND diets

| 0.5% | 1.1% |

B

TUNEL positive nuclei

$P = 0.062$

TUNEL positive nuclei, %

Ca concentration in LND diets

C

Caspase 3

$P = 0.066$

Relative mRNA expression

Ca concentration in LND diets

Fig. 7. Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) positive nuclei labeled by staining. (A) TUNEL photographs of apoptotic nuclei of duck meat in the low nutrient density (LND) diet with 0.5% and 1.1% Ca (200× magnification). (B) Percentage of positive nuclei per whole muscle section. (C) The transcription of *caspase 3* in duck meat were quantified by RT-PCR. Data represent means with standard deviation.

Author contributions

Huaiyong Zhang: Animal trial, Data collection and evaluation, Laboratory and Statistical analysis, Writing; **Quifeng Zeng:** Methodology, Data collection and evaluation, Laboratory analysis, Writing; **Shiping Bai**: Data collection and evaluation, Statistical analysis, and Writing; **Jianping Wang:** Data evaluation, Manuscript review, Writing; **Xuemei Ding:** Data evaluation, Manuscript review; **Yue Xuan:** Animal trial, Laboratory analysis, Data collection; **Zhuowei Su:** Animal trial, Laboratory analysis, Data collection; **Joris Michiels:** Data evaluation, Statistical analysis, Critical manuscript review; **Keying Zhang:** Study design, Feed formulation, Data evaluation, Critical manuscript review.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.10.005.

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