British Journal of Nutrition, page 1 of 16 doi:10.1017/S0007114524001181

The amino acid sensor methionyl-tRNA synthetase is required for methionine-induced milk protein synthesis in a domestic pigeon model

Panpan Lu[1,2], Chen Zhong[1], Hongwei Zhao[1], Fuyong Li[3], Xiaofan Wang[1], Xiuqi Wang[1], Huichao Yan[1] and Chunqi Gao[1]*

[1]*College of Animal Science, Guangdong Provincial Key Laboratory of Animal Nutrition Control/Guangdong Laboratory for Lingnan Modern Agriculture/State Key Laboratory of Swine and Poultry Breeding Industry, South China Agricultural University, Guangzhou 510642, People's Republic of China*
[2]*Henry Fork School of Biology and Agriculture, Shaoguan University, Shaoguan 512005, People's Republic of China*
[3]*Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong SAR, People's Republic of China*

(Submitted 5 October 2023 – Final revision received 16 February 2024 – Accepted 13 March 2024)

Abstract

This study was conducted to investigate whether methionyl-tRNA synthetase (MetRS) is a mediator of methionine (Met)-induced crop milk protein synthesis via the janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) signalling pathway in breeding pigeons. In Experiment 1, a total of 216 pairs of breeding pigeons were divided into three groups (control, Met-deficient, and Met-rescue groups). In Experiments 2 and 3, forty pairs of breeding pigeons from each experiment were allocated into four groups. The second experiment included a control group and three MetRS inhibitor (REP8839) groups. The third experiment included a Met-deficient group, Met-sufficient group, REP8839 + Met-deficient group and REP8839 + Met-sufficient group. Experiment 1 showed that Met supplementation increased crop development, crop milk protein synthesis, the protein expression of MetRS and JAK2/STAT5 signalling pathway, and improved squab growth. Experiment 2 showed that crop development, crop milk protein synthesis and the protein expression of MetRS and the JAK2/STAT5 signalling pathway were decreased, and squab growth was inhibited by the injection of 1·0 mg/kg body weight REP8839, which was the selected dose for the third experiment. Experiment 3 showed that Met supplementation increased crop development, crop milk protein synthesis and the expression of MetRS and JAK2/STAT5 signalling pathway and rescued squab growth after the injection of REP8839. Moreover, the co-immunoprecipitation results showed that there was an interaction between MetRS and JAK2. Taken together, these findings indicate that MetRS mediates Met-induced crop milk protein synthesis via the JAK2/STAT5 signalling pathway, resulting in improved squab growth in breeding pigeons.

Keywords: **Breeding pigeon: Methionine: Crop milk protein synthesis: Molecular mechanism**

Pigeons are altricial birds, and newly hatched pigeon squabs cannot eat independently and are fed by their parents from mouth to mouth with a 'curd-like substance' called crop milk during the early period after hatching. In particular, they receive pure crop milk to live in the first week after hatching, after which the crop milk is gradually replaced by an adult diet. In contrast to mammalian milk, crop milk is synthesised by both female and male pigeons from the germinal epithelium of the crop sac from 14 d of brooding to 28 d of lactation[1]. The nutritional status of breeding pigeons directly determines the quality of crop milk secreted by their crop.

The quality of crop milk plays an important role in the survival, growth and development of squabs[2]. The main nutritional ingredient of crop milk is protein (64 %), 90 % of which is casein[3,4]. The production of crop milk depends on the proliferation of crop epithelial cells and their milk synthesis ability. Several studies have reported that essential amino acids stimulate cell proliferation[5,6] and can regulate casein synthesis

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; ADW, average daily weight ; BW, body weight; BWG, body weight gain; BWL, body weight loss; Co-IP, co-immunoprecipitation; JAK2, janus kinase 2; KRT19, Keratin 19; Met, methionine; MetRS, methionyl-tRNA synthetase; PCNA, proliferating cell nuclear antigen; p-JAK2, phosphorylated janus kinase 2; p-STAT5, phosphorylated signal transducer and activator of transcription 5; SOCS3, suppressor of cytokine signalling 3; STAT5, signal transducer and activator of transcription 5.

* **Corresponding author:** Dr C. Gao, fax 86-20-38882017, email cqgao@scau.edu.cn

in bovine mammary gland epithelial cells via the janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) pathway[7,8]. Methionine (Met) has been identified as the first limiting amino acid for birds and plays an important role in protein synthesis[9]. Dietary supplementation with Met can increase milk production in cows[10,11]. Conversely, Met deficiency impairs milk protein synthesis and results in growth retardation[12,13]. Previous studies reported that Met increased the transcription and translation of milk protein genes via the JAK2/STAT5 signalling pathway[14,15]. Our earlier study also revealed that the addition of Met to breeding pigeons regulated crop milk protein synthesis via the JAK2/STAT5 signalling pathway and improved the growth performance of squabs[16]. However, how Met stimulates the JAK2/STAT5 signalling pathway for crop milk protein synthesis is unclear.

Aminoacyl-tRNA synthetase is a novel signalling protein that may have noncanonical functions unrelated to its catalytic activity in protein synthesis. Aminoacyl-tRNA synthetase with sufficient amino acids can sense environmental signals and modulate cellular functions[17,18]. Early studies have proven that glycyl-tRNA synthetase (GlyRS) and seryl-tRNA synthetase (SerRS) mediate amino acid synthesis and enhance milk protein synthesis in bovine mammary epithelial cells[19,20]. On the basis of our previous work, we speculated that methionyl-tRNA synthetase (MetRS) may be a potential mediator of Met-induced crop milk protein synthesis in breeding pigeons. However, the mechanism by which MetRS mediates Met-induced crop milk protein synthesis is unknown.

Milk is an ideal form of nutrition for the growth and development of infants or newborn animals. The nutrient quality of milk is of great concern to researchers. Therefore, the objective of this study was to investigate whether MetRS mediates Met-induced crop milk protein synthesis via the JAK2/STAT5 signalling pathway and improves squab growth by using domestic pigeons as a model.

Materials and methods

Ethical statement

All procedures used in this study were approved by the Animal Care Advisory Committee of South China Agricultural University (Guangzhou, China).

Experimental design

Three experiments were conducted in this study. All white king breeding pigeons were obtained from Baifeng pigeon farm (Jiangmen, China). The experimental design was as follows:

Experiment 1. A total of 216 pairs of breeding pigeons with similar body weights (BW), productive performances and egg-laying habits were randomly divided into three treatment groups of six replicates, and each replicate included twelve pairs of breeding pigeons. DL-Met (Met Amino, >99 %, Evonik Degussa GmbH, Essen, Germany) was used in this study. The control group was fed a basal diet containing 0·55 % Met throughout the experimental period. Two experimental groups were fed a Met-deficient diet containing 0·21 % Met from the raw material throughout the experimental period and not supplemented with synthetic Met (Met deficient), and one group of birds was fed a Met-deficient diet from 1 to 13 d of the experimental period and then basal diet from 14 to 45 d of the experimental period (Met rescue). This experiment lasted for 45 d, including the incubation and lactation periods of 17 and 28 d, respectively.

Experiment 2. A total of forty pairs of breeding pigeons with similar productive performance and egg laying on the same day were allocated into four treatment groups of ten pairs of breeding pigeons. The treatment groups were the control group (injected with normal saline) and the three MetRS inhibitor groups (injected with 0·5, 1·0 or 1·5 mg/kg REP8839). On the 1st, 3rd, 5th and 7th days of the lactation period, the birds were injected with normal saline or different doses of REP8839 through the wing vein. This experiment started on the 1st day of the lactation period and lasted for 7 d.

REP8839 solutions were prepared as follows: REP8839 powder (Axon MedChem, Groningen, Netherlands) was dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. The REP8839-DMSO solution was added to normal saline to prepare a 3 mg/ml REP88339 solution, which was further diluted to 2 mg/ml and 1 mg/ml, corresponding to 1·5, 1·0 and 0·5 mg/kg BW of REP8839, respectively. The injection volume was 300 μl per breeding pigeon (600 g BW).

Experiment 3. The inhibitor (1·0 mg/kg REP8839) from Experiment 2 was used for the following experiment. A total of forty pairs of breeding pigeons with similar productive performance and egg laying on the same day were divided into four treatment groups with ten pairs of breeding pigeons. The treatment groups consisted of the Met-deficient group (injected with saline + Met-deficient diet), the Met-sufficient group (injected with saline + Met-sufficient diet), the REP8839 + Met-deficient group (injected with 1·0 mg/kg REP8839 + Met-deficient diet) and the REP8839 + Met-sufficient group (injected with 1·0 mg/kg REP8839 + Met-sufficient diet). The four groups were injected with 300 μl of normal saline or 1·0 mg/kg REP8839 on the 1st, 3rd, 5th or 7th day of the lactation period. This experiment lasted for 7 d.

Management and diets

Each pair of breeding pigeons with four squabs was reared in the same cage, all pigeons were housed in three-tiger cages in an environmentally controlled room and light was maintained for 16 h daily throughout the experiment. The squabs were fed crop milk from their parent pigeons. Feed and water were available *ad libitum*. The feed was a pea–corn–sorghum formulation and met or exceeded the nutrient requirements of pigeons according to the Poultry Nutrient Requirement guidelines. The ingredients and nutritional composition of the basal diet are shown in Online Supplementary Table S1.

Performance measurements

The feed intake of each breeding pigeon pair was recorded daily to calculate the average daily feed intake (ADFI) for incubation, lactation and the entire experimental period.

At the beginning and end of the incubation and lactation periods, the BW of each breeding pigeon pair was recorded to calculate the BW gain (BWG) for the incubation period and the BW loss (BWL) for the lactation and the entire experimental period. The egg-laying interval of the female pigeons was considered the day after the first egg was laid in the first clutch to the day before the first egg was laid in the subsequent clutch.

The BW of the squabs was recorded on the 1st, 7th, 14th, 21st and 28th days after hatching. The average daily gain (ADG) of the squabs was calculated.

Sample collection

In Experiment 1, all of the birds were fasted for 12 h on the 24th and 31st days of the experiment (7th and 14th days of the lactation period), respectively, and thirty-six pairs of breeding pigeons with similar BWs were selected (twelve pairs of breeding pigeons per treatment) and euthanised by cervical dislocation. In Experiments 2 and 3, forty pairs of breeding pigeons were selected on the 8th day of the experimental period and euthanised by cervical dislocation.

Crop milk was collected from the crop tissue of the breeding pigeons selected from three experiments, immediately frozen in liquid nitrogen and stored at –80°C. The isolated crop tissue was washed with PBS and weighed. The crop weight was calculated based on the BW. The crop thickness was measured via three sections randomly selected from the crop tissue. The crop tissue was partly stored at –80°C for subsequent analysis and partly preserved in 4 % paraformaldehyde for immunofluorescence staining.

Casein-level determination

The levels of αS1-casein, αS2-casein and β-case in crop milk were determined using ELISA kits (Enzyme-linked Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's instructions.

Western blotting analysis

Protein was extracted from the crop tissue as previously described[21,22]. In brief, the protein concentration in the crop tissue was measured using a bicinchoninic acid protein assay kit (Applygen Technology, Inc., Beijing, China). Equal amounts of protein were separated by 10 % SDS–PAGE, transferred to polyvinylidene difluoride membranes (PVDF, 0·45 μm; Millipore, Billerica, MA, USA) and blocked with 5 % non-fat milk in TBST (0·05 % Tween 20, 100 mM Tris-HCl, 150 mM NaCl, pH 7·4) for 2 h. The membranes were then incubated with primary antibodies at 4°C overnight (online Supplementary Table S2). Then, the PVDF membranes were incubated with secondary antibodies for 1 h. Finally, the immunological signals were detected with enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology) in a FluorChem M system (Cell Biosciences, San Leandro, CA, USA). The bands were quantified using image analysis software (Tanoan, Shanghai, China).

Immunofluorescence analysis

Crop tissues were removed from 4 % paraformaldehyde, rinsed with PBS, dehydrated with graded ethanol, cleared in xylene and embedded in paraffin according to previous methods, with slight modifications[23]. 5 μm thick sections of the crop tissue were obtained for immunofluorescence staining. The sections were incubated in blocking solution containing 5 % bovine serum albumin at room temperature for 2 h. After cleaning, the sections were incubated with primary antibodies overnight and rinsed three times. Each rinse lasted 5 min in PBS. Secondary antibodies were then added to the sections for 2 h, followed by rinsing three times. Tissues were treated with 40,6-diamidino-2-phenylindole to stain the nuclei, incubated for 10 min at room temperature and then rinsed three times with PBS. The fluorescence images were examined using a fluorescence microscope (NIS-Elements, Nikon, Tokyo, Japan).

Co-immunoprecipitation assay

Proteins from the crop tissue were immunoprecipitated using a Pierce Co-IP Kit (Epizyme Biomedical Technology Co. Ltd., China). Briefly, protein was extracted using lysis buffer and centrifugation. The extracted protein was incubated with antibody-MetRS and protein A/G magnetic beads overnight at 4°C to form protein complexes and then eluted with elution buffer. Further details were followed according to the manufacturer's instructions. The eluted protein was separated by SDS–PAGE and subjected to Western blotting. The proteins that interacted with the bait proteins were precipitated by co-IP using the MetRS antibody as bait and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a negative control.

Statistical analysis

All of the data were analysed using GraphPad Prism version 8·0 (GraphPad Software, Inc., San Diego, CA) for statistical analysis via one-way ANOVA. Significant differences between treatments were measured using Tukey's multiple comparison test. All of the results are presented as the mean and standard error of the mean (SEM). P values $< 0{\cdot}05$ were considered to indicate statistical significance.

Results

Experiment 1

Methionine supplementation promotes the productive performance of breeding pigeons and squab growth. First, we determined the effects of Met supplementation on the productive performance of breeding pigeons and squab growth. No significant difference was found ($P > 0{\cdot}05$) in the ADFI between the incubation and lactation periods in the Met-deficient group (Fig. 1(a) and (b)), but the ADFI was significantly greater ($P < 0{\cdot}05$) throughout the whole experimental period (Fig. 1(c)). The BWG of the breeding pigeons decreased ($P < 0{\cdot}05$) during the incubation period (Fig. 1(d)), and the BWL increased ($P < 0{\cdot}05$) during the lactation and whole experimental periods (Fig. 1(e) and (f)) in the Met-deficient group. The egg-laying interval was also extended ($P < 0{\cdot}05$) in

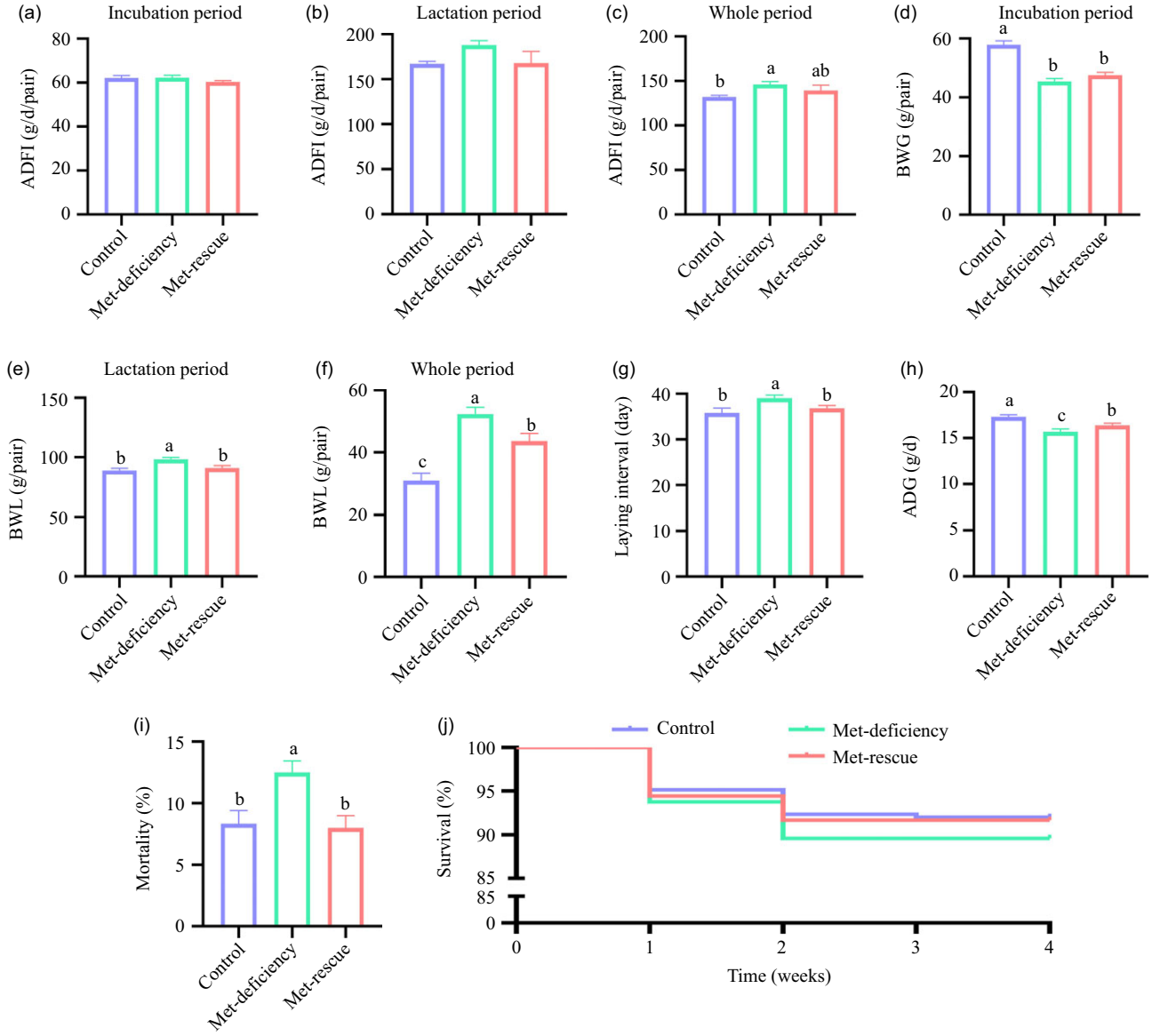

Fig. 1. Effects of methionine (Met) supplementation on the productive performance of breeding pigeons and squab growth. (a) Average daily feed intake (ADFI) of breeding pigeons during the incubation period. (b) ADFI of breeding pigeons during the lactation period. (c) ADFI of breeding pigeons during the whole experimental period. (d) Body weight gain (BWG) of breeding pigeons during the incubation period. (e) Body weight loss (BWL) of breeding pigeons during the lactation period. (f) BWL of breeding pigeons during the whole experimental period. (g) Laying interval of breeding pigeons. (h) Average daily gain (ADG) of squabs. (i) Mortality rate of squabs. (j) Survival rate of the squabs over 4 weeks. The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0{\cdot}05$, n=6).

the Met-deficient group (Fig. 1(g)). After the administration of Met to the breeding pigeons, BWL decreased ($P < 0{\cdot}05$) during lactation and throughout the entire experimental period, and the egg-laying interval of the breeding pigeons decreased ($P < 0{\cdot}05$). These results indicate that sufficient Met intake is necessary for breeding pigeons for nutritional reasons to support reproductive performance.

As shown in Fig. 1(h) and (i), compared with the control diet, the Met-deficient diet significantly decreased the ADG of squabs and increased ($P < 0{\cdot}05$) the mortality rate. As expected, Met supplementation increased the ADG ($P < 0{\cdot}05$) and decreased ($P < 0{\cdot}05$) the mortality rate of squabs. In addition, the survival rate of the squabs increased after hatching in response to supplementation with Met (Fig. 1(j)). These findings suggested that supplementation with Met can effectively remedy the poor growth of squabs.

Methionine supplementation improves crop development and crop milk protein synthesis. We further determined the effects of Met supplementation on crop development and crop milk protein synthesis. Compared with the control diet, the Met-deficient diet significantly decreased the weight and thickness of the crop ($P < 0{\cdot}05$) in the breeding pigeons at 7 and 14 d after hatching, respectively (Fig. 2(a)–(d)). The levels of αS1-casein, αS2-casein and β-casein (Fig. 2(e)) were lower ($P < 0{\cdot}05$) in the Met-deficient group than in the control group. In addition, the

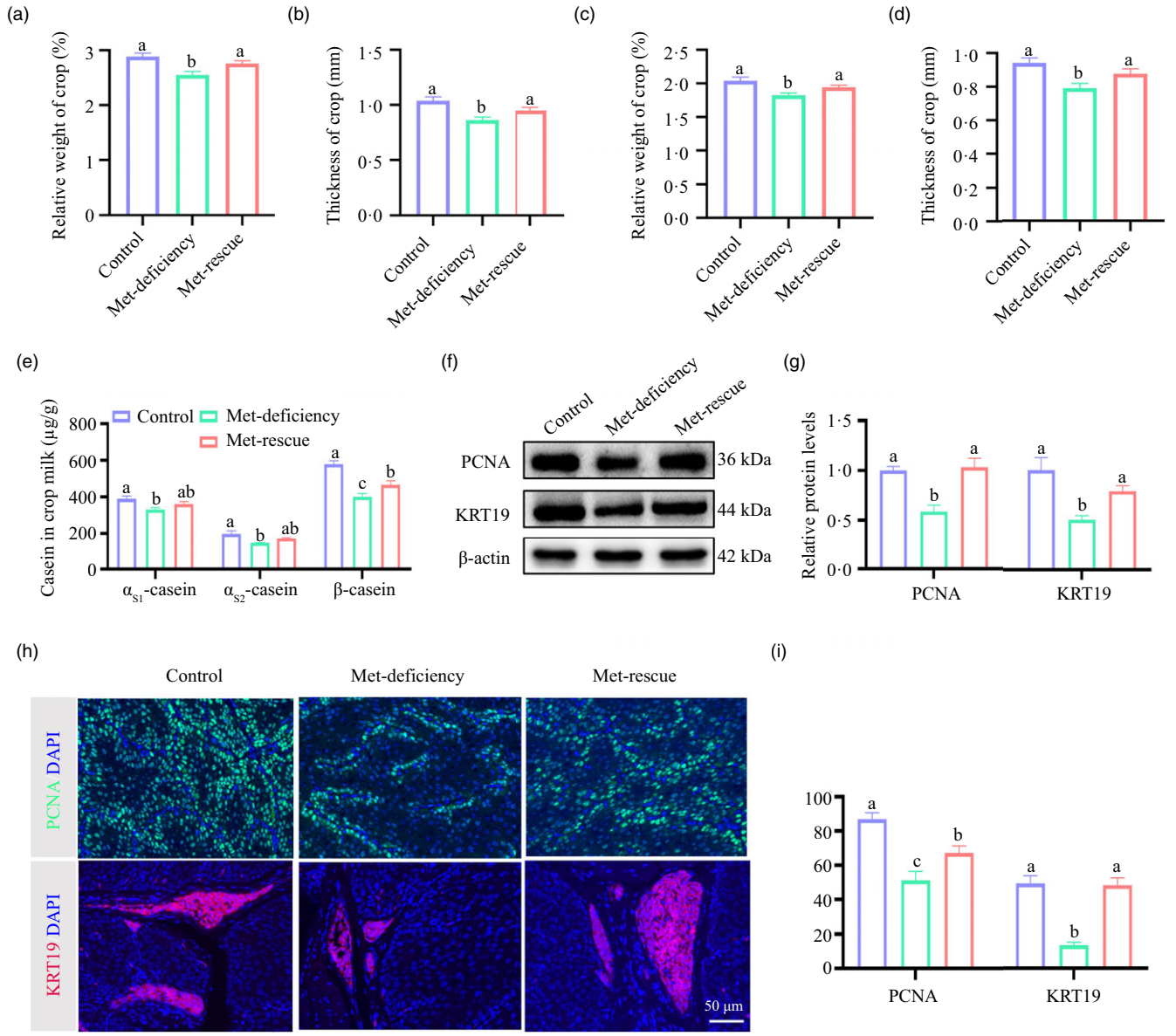

Fig. 2. Effects of methionine (Met) supplementation on crop development, levels of crop milk protein and proliferation and differentiation of crop epithelial cells in breeding pigeons. (a) Relative weight of the crop on the 7th day of the lactation period ($n = 6$). (b) Thickness of the crop on the 7th day of the lactation period ($n = 6$). (c) Relative weight of the crop on the 14th day of the lactation period ($n = 6$). (d) Thickness of the crop on the 14th day of the lactation period ($n = 6$). (e) Contents of α_{S1}-casein, α_{S2}-casein and β-casein in crop milk ($n = 6$). (f) Western blot analysis of proliferating cell nuclear antigen (PCNA) and Keratin 19 (KRT19). (g) Densitometric quantification of PCNA and KRT19 ($n = 3$). (h) Representative images of immunofluorescence staining for PCNA and KRT19 (scale bar = 50 μm). (i) Fluorescence intensity of PCNA and KRT19 ($n = 3$). The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0{\cdot}05$).

protein expression and immunofluorescence signal intensities of proliferating cell nuclear antigen (PCNA) and Keratin 19 (KRT19) were lower ($P < 0{\cdot}05$) in the Met-deficient group than in the control group (Fig. 2(f)–(i)). As expected, Met supplementation improved ($P < 0{\cdot}05$) the weight and thickness of the crops of the breeding pigeons at 7 and 14 d after hatching, respectively. The level of β-casein was also increased ($P < 0{\cdot}05$) by Met supplementation. Moreover, protein expression and fluorescence signal intensities of PCNA and KRT19 were significantly increased ($P < 0{\cdot}05$) by Met supplementation. These results suggest that supplementation with Met effectively improves crop morphology and the secretion of crop milk protein, possibly through the proliferation and differentiation of crop epithelial cells.

Methionine supplementation increases methionyl-tRNA synthetase expression and improves crop milk protein synthesis by activating the janus kinase 2/signal transducer and activator of transcription 5 signalling pathway. We further investigated whether Met stimulates MetRS expression and promotes crop milk protein synthesis via the JAK2/STAT5 signalling pathway. As shown in Fig. 3(a) and (b), compared with those in the control group, the protein expression of MetRS and the ratios of phosphorylated JAK2 (p-JAK2) to JAK2 and phosphorylated STAT5 (p-STAT5) to STAT5 were decreased ($P < 0{\cdot}05$). In addition, suppressor of cytokine signalling 3 (SOCS3) protein expression was upregulated ($P < 0{\cdot}05$) in the Met-deficient group, and the protein

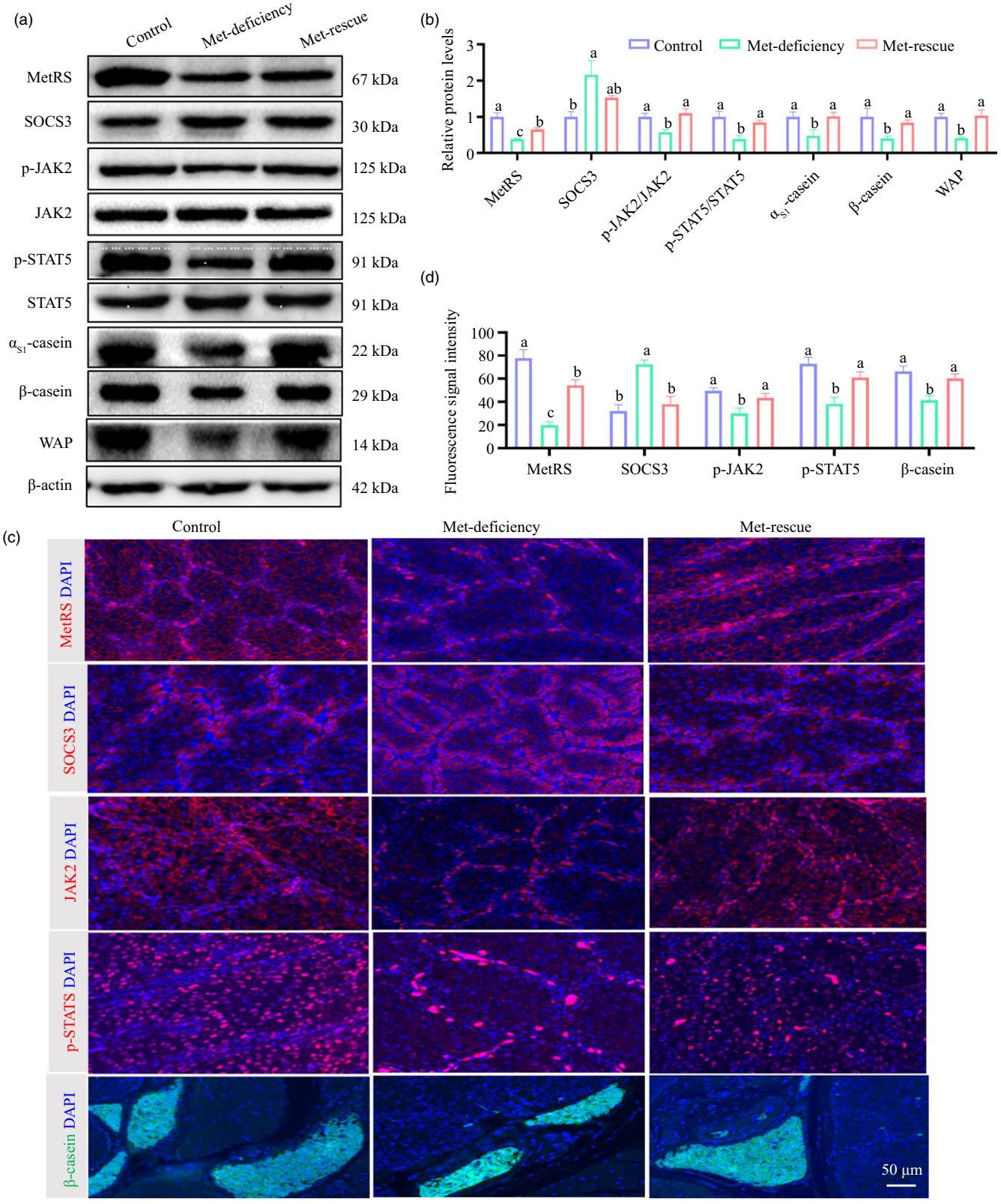

Fig. 3. Effects of methionine (Met) supplementation on methionyl-tRNA synthetase (MetRS), the JAK2/STAT5 signalling pathway and crop milk protein synthesis in breeding pigeons. (a) Western blot analyses of MetRS, suppressor of cytokine signalling 3 (SOCS3), phosphorylated janus kinase 2 (p-JAK2), JAK2, phosphorylated signal transducer and activator of transcription 5 (p-STAT5), STAT5, αs1-casein, β-casein and whey acidic protein (WAP). (b) Densitometric quantification of MetRS, SOCS3, p-JAK2/JAK2, p-STAT5/STAT5, αs1-casein, β-casein and WAP. (c) Representative images of immunofluorescence staining for MetRS, SOCS3, p-JAK2, p-STAT5 and β-casein (scale bar = 50 μm). (d) Fluorescence intensity of MetRS, SOCS3, p-JAK2/JAK2, p-STAT5/STAT5 and β-casein. The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0{\cdot}05$, n 3).

British Journal of Nutrition

expression of αs1-casein and whey acidic protein (WAP) was also downregulated ($P < 0·05$). Similarly, the fluorescence signal intensities of MetRS, SOCS3, p-JAK2, p-STAT5 and β-casein were significantly lower ($P < 0·05$) in the Met-deficient group (Fig. 3(c) and (d)). As expected, Met supplementation improved ($P < 0·05$) the expression and immunofluorescence signal intensities of these proteins. These results suggest that sufficient Met stimulates MetRS expression and activates the JAK2/STAT5 signalling pathway, leading to an increase in crop milk protein synthesis.

Experiment 2

REP8839 does not affect the productive performance of breeding pigeons but hinders squab growth. We conducted this experiment to investigate the effects of different concentrations of REP8839 on the productive performance of breeding pigeons and squab growth. The results showed that the injection of different concentrations of REP8839 did not cause significant differences ($P > 0·05$) in the ADFI or BWL between breeding control group and REP8839-treated groups (Fig. 4(a) and (b)). In addition, compared with the control, ERP8839 injection at 1·0 and 1·5 mg/kg BW over 7 d resulted in a decrease ($P < 0·05$) in the average daily weight (ADW) of squabs and an increase ($P < 0·05$) in the mortality rate of squabs during lactation (Fig. 4(c) and (d)). The survival rate was lowest at 1·0 mg/kg BW of REP8839 after 7 d of lactation (Fig. 4(e)). These results indicate that the injection of REP8839 has no negative effect on the productive performance of breeding pigeons but negatively affects squab growth at 1·0 and 1·5 mg/kg. BW, respectively.

REP8839 inhibits crop development and crop milk protein synthesis. The effects of REP8839 on crop development and crop milk protein synthesis were further investigated. Fig. 5(a) and (b) shows that the injection of REP8839 (1·0 and 1·5 mg/kg. BW) significantly decreased ($P < 0·05$) the weight and thickness of the crop compared with those of the control group. Similarly, the levels of αS1-casein, αS2-casein and β-casein were significantly lower ($P < 0·05$) in the 0·5, 1·0 and 1·5 mg/kg BW REP8839 groups, respectively, than in the control group (Fig. 5(c)). Among them, the protein levels of αS1-casein and β-casein were lower ($P < 0·05$) in the 1·0 and 1·5 mg/kg BW REP8839 groups, respectively, whereas the protein level of αS2-casein was the lowest ($P < 0·05$) in the 1·5 mg/kg BW REP8839 group. As shown in Fig. 5(d)–(g), injection of REP8839 at 0·5, 1·0 and 1·5 mg/kg BW significantly decreased ($P < 0·05$) PCNA protein expression in the crop epithelial cells of breeding pigeons compared with that in crop epithelial cells of the control breeding pigeons, and the fluorescence signal intensity of PCNA was decreased ($P < 0·05$) after 1·0 and 1·5 mg/kg BW REP8839 injection. The protein expression and fluorescence signal intensity of KRT19 were decreased ($P < 0·05$) by 1·0 and 1·5 mg/kg BW of REP8839 in the crop epithelial cells of breeding pigeons, respectively, compared with those in the control group. This finding indicates that 1·0 and 1·5 mg/kg BW of REP8839 could be the optimal dose for effectively inhibiting crop development and the proliferation and differentiation of crop epithelial cells, resulting in a decrease in crop milk protein synthesis.

REP8839 inhibits methionyl-tRNA synthetase expression and crop milk protein synthesis by blocking the janus kinase 2/signal transducer and activator of transcription 5 signalling pathway. We explored whether REP8839 injection inhibits MetRS expression and crop milk protein synthesis by blocking the JAK2/STAT5 signalling pathway. As shown in Fig. 6(a) and (b), REP8839 at 1·0 and 1·5 mg/kg BW significantly decreased ($P < 0·05$) MetRS protein expression, the ratios of p-JAK2 to JAK2 and p-STAT to STAT5 and the protein expression of β-casein and WAP in the crop epithelial cells of breeding pigeons. Similarly, the protein expression of αS1-casein was decreased ($P < 0·05$) at 1·5 mg/kg REP8839 in the crop epithelial cells of breeding pigeons, whereas SOCS3 protein expression was increased ($P < 0·05$) at 1·0 and 1·5 mg/kg BW of REP8839 in the crop epithelial cells of breeding pigeons. As shown in Fig. 6(c) and (d), the fluorescence signal intensities of MetRS and β-casein decreased ($P < 0·05$) by 0·5, 1·0 and 1·5 mg/kg BW REP8839 in the crop epithelial cells of breeding pigeons, and the fluorescence signal intensities of SOCS3, p-JAK2 and p-STAT5 were consistent ($P < 0·05$) with the protein expression results. These results suggest that REP8839 inhibits MetRS expression and blocks the JAK2/STAT5 signalling pathway, leading to a decrease in crop milk protein synthesis.

Experiment 3

To investigate whether MetRS mediates Met-induced crop milk protein synthesis via the JAK2/STAT5 signalling pathway. We performed this experiment by supplementing Met in combination with the inhibitor REP8839.

Methionine supplementation does not affect the productive performance of breeding pigeons but relieves the inhibition of squab growth induced after the injection of REP8839. We observed the effects of Met on the productive performance of breeding pigeons and squab growth after the injection of REP8839. As shown in Fig. 7(a) and (b), no significant differences were found ($P > 0·05$) in the ADFI or BWL among the breeding pigeons in any of the groups. However, the ADW of the squabs was significantly greater ($P < 0·05$) after 7 d, and the mortality rate was lower ($P < 0·05$) in the REP8839 group with Met supplementation than in the REP8839 group without Met supplementation (Fig. 7(c) and (d)). Consistently, the survival rate increased in response to the injection of REP8839 in combination with Met supplementation (Fig. 7(e)). These findings indicate that Met supplementation has no effect on the productive performance of breeding pigeons but relieves the inhibition of squab growth after the injection of REP8839.

Methionine supplementation rescues the inhibition of crop development and crop milk protein synthesis after the injection of REP8839. We further investigated the effects of Met on crop development and crop milk protein synthesis in breeding pigeons after the injection of REP8839. We found that the weight and thickness of the crop were significantly greater

British Journal of Nutrition

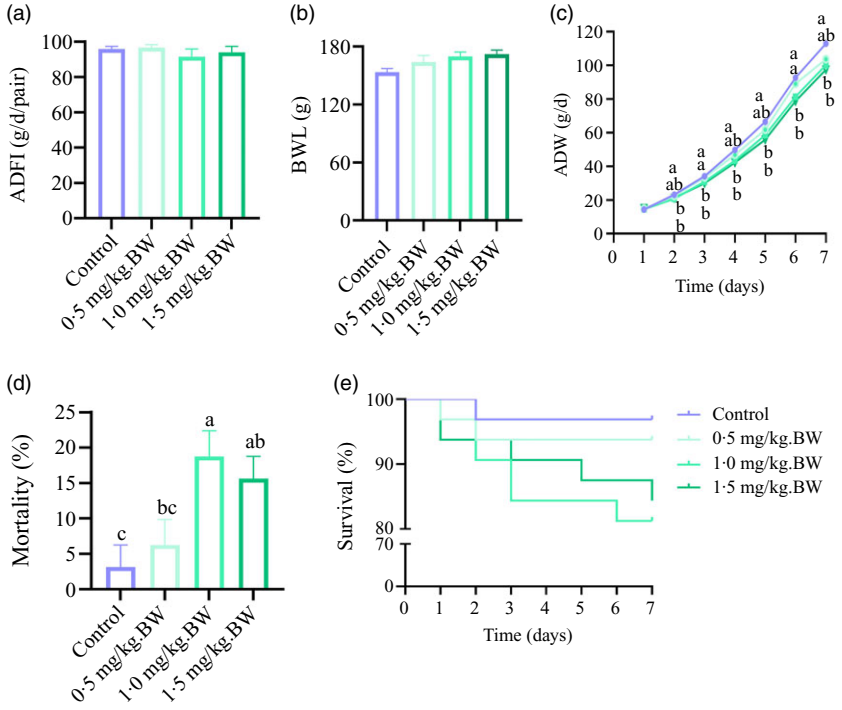

Fig. 4. Effects of different levels of REP8839 on the productive performance of breeding pigeons and squab growth. (a) Average daily feed intake (ADFI) of breeding pigeons. (b) Body weight loss (BWL) of breeding pigeons. (c) Average daily weight (ADW) of squabs over 7 d during lactation. (d) Mortality rate of squabs. (e) The survival rate of the squabs over 7 d during lactation. The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0·05$, n 10).

($P < 0·05$) in the REP8839 + Met treatment group than in the REP8839 group without Met supplementation (Fig. 8(a) and (b)). Moreover, Met supplementation increased ($P < 0·05$) the levels of αS1-casein and β-casein after the injection of REP8839 (Fig. 8(c)). In addition, Met supplementation significantly increased ($P < 0·05$) the protein expression of PCNA and KRT19 after the injection of REP8839 compared with that in the REP8839 group without Met supplementation (Fig. 8(d) and (e)). Similarly, the fluorescence signal intensities of PCNA and KRT19 had similar results ($P < 0·05$) to the protein expression results (Fig. 8(f) and (g)). These results imply that Met supplementation rescues the inhibition of crop development and increases the level of crop milk casein via crop cell proliferation in breeding pigeons.

Methionine supplementation blocks the inhibition of methionyl-tRNA synthetase expression and activates the janus kinase 2/signal transducer and activator of transcription 5 signalling pathway after the injection of REP8839. The underlying mechanism was further explored to determine whether MetRS responds to Met stimulation and improves crop milk protein synthesis by activating the JAK2/STAT5 signalling pathway. As shown in Fig. 9(a) and (b), Met supplementation increased MetRS protein expression, the ratio of p-JAK2 to JAK2 and p-STAT5 to STAT5 and the protein expression of β-casein and WAP, whereas SOCOS3 protein expression was decreased ($P < 0·05$) after injection of REP8839 compared with that in the REP8839 group without Met supplementation. The fluorescence signal intensities of MetRS, SOCS3, p-JAK2, p-STAT5 and β-casein were consistent with the protein expression results ($P < 0·05$) (Fig. 9(c) and (d)). These results indicate that Met stimulates MetRS and activates the JAK2/STAT5 signalling pathway, leading to an increase in crop milk protein synthesis.

Methionyl-tRNA synthetase interacts with janus kinase 2 and regulates its activity. Using co-IP, we further investigated the colocalisation of MetRS with SOCS3 and JAK2 in crops. The results showed that JAK2 interacted with MetRS (Fig. 9(f)), whereas SOCS3 did not bind to MetRS (Fig. 9(e)). These findings suggested that MetRS might trigger JAK2 activity.

Discussion

The health status and productivity of breeding pigeons during both the incubation and lactation periods are influenced mainly by nutrients. Met is the first limiting amino acid, and Met deficiency may lead to disastrous results in avian species, including impaired protein synthesis and growth depression or productivity loss in poultry[24–26]. The present study showed that Met deficiency (containing 0·21 % Met in the raw feed material) resulted in an increase in BWL during lactation, throughout the entire experimental period, and in the egg-laying interval, while the BWG of breeding pigeons decreased during the incubation period. However, as expected, the productive performance of the breeding pigeons improved after Met supplementation (0·3%). Similarly, early studies from our laboratory demonstrated that Met supplementation enhanced the ADG and decreased the BWL of breeding pigeons[13]. A previous study also revealed that

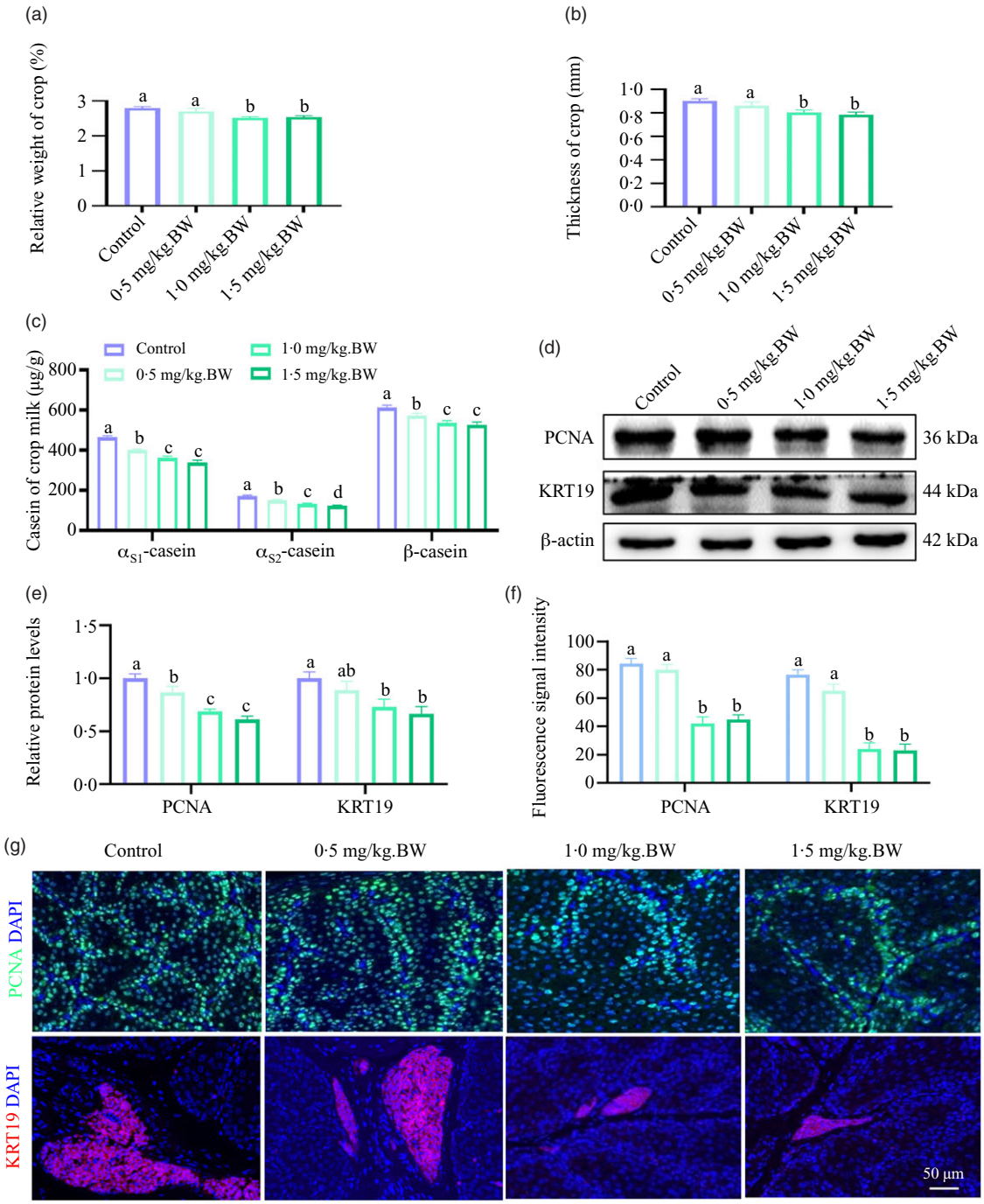

Fig. 5. Effects of different concentrations of REP8839 on crop development, crop milk protein levels and proliferation and differentiation of crop epithelial cells in breeding pigeons. (a) Relative weight of the crop ($n = 10$). (b) Thickness of the crop ($n = 10$). (c) Levels of α_{S1}-casein, α_{S2}-casein and β-casein in crop milk ($n = 10$). (d) Western blot analysis of proliferating cell nuclear antigen (PCNA) and Keratin 19 (KRT19). (e) Densitometric quantification of PCNA and KRT19 ($n = 3$). (f) Fluorescence intensity of PCNA and KRT19 ($n = 3$). (g) Representative images of immunofluorescence staining for PCNA and KRT19 (scale bar = 50 μm). The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0·05$).

BWG of chickens fed a Met-sufficient diet was greater than that of chickens fed a Met-deficient diet[27]. It has been reported that the nutrient requirements of cows gradually increase during the lactation period. When dietary nutrients are not sufficient to meet these requirements, a negative energy balance and a decrease in productive performance may occur[28]. Therefore, our results can be explained by the fact that Met supplementation met the amino acid requirements of breeding pigeons and blocked protein breakdown, rescued BWL during the lactation period and BWG during the incubation period, and shortened the egg-laying interval. Remarkably, Met deficiency increased the ADFI of the breeding pigeons in this study. Normally, an increase in feed intake is accompanied by an increase in the Met concentration[26,29]. A previous study indicated that the feed

British Journal of Nutrition

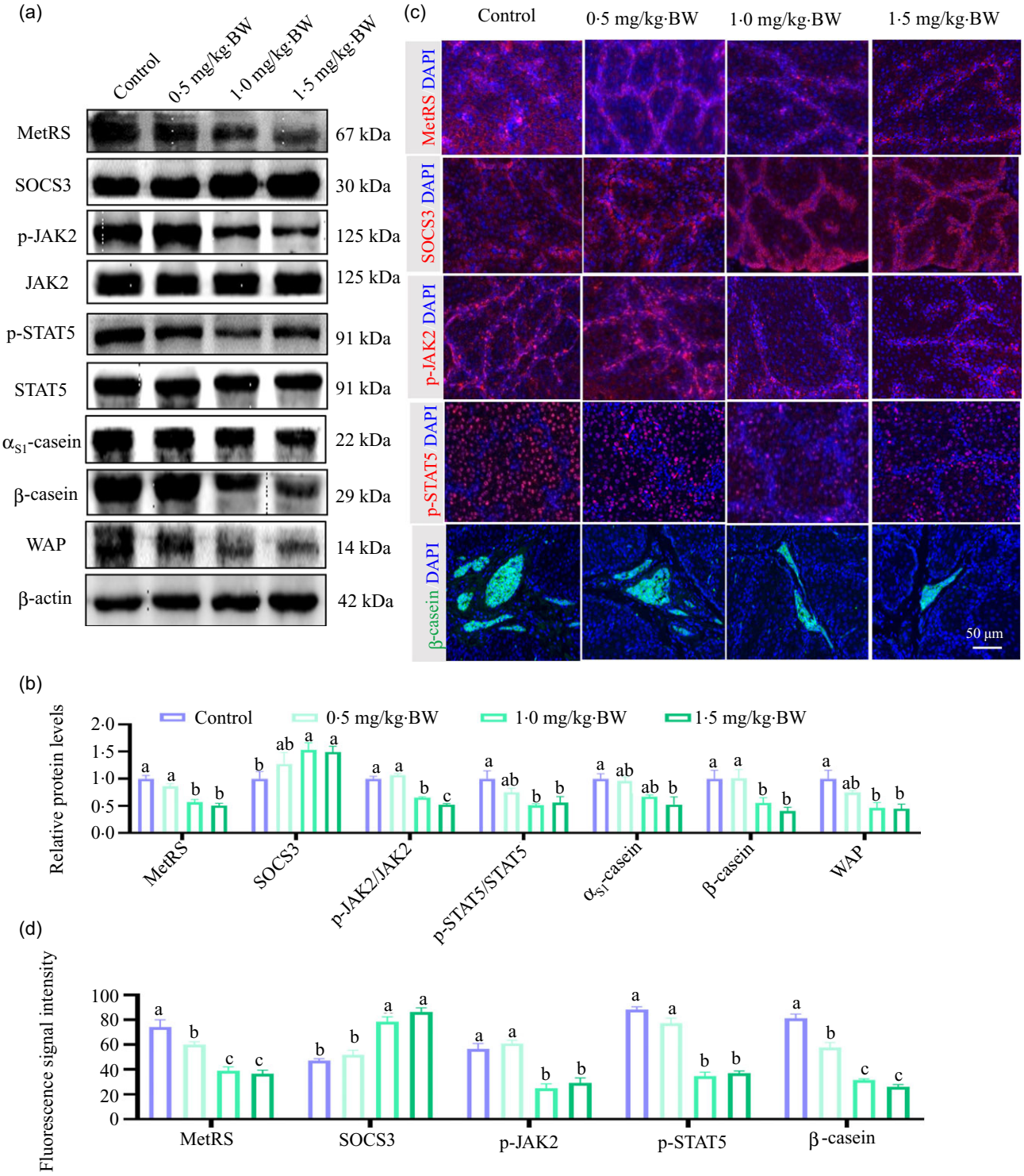

Fig. 6. Effects of different levels of REP8839 on methionyl-tRNA synthetase (MetRS), the JAK2/STAT5 signalling pathway and crop milk protein synthesis in breeding pigeons. (a) Western blot analyses of MetRS, suppressor of cytokine signalling 3 (SOCS3), phosphorylated janus kinase 2 (p-JAK2), JAK2, phosphorylated signal transducer and activator of transcription 5 (p-STAT5), STAT5, αs1-casein, β-casein and whey acidic protein (WAP). (b) Densitometric quantification of MetRS, SOCS3, p-JAK2/JAK2, p-STAT5/STAT5, αs1-casein, β-casein and WAP. (c) Representative images of immunofluorescence staining for MetRS, SOCS3, p-JAK2, p-STAT5 and β-casein (scale bar = 50 μm). (d) Fluorescence intensity of MetRS, SOCS3, p-JAK2, p-STAT5 and β-casein. The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0{\cdot}05$, n 3).

British Journal of Nutrition

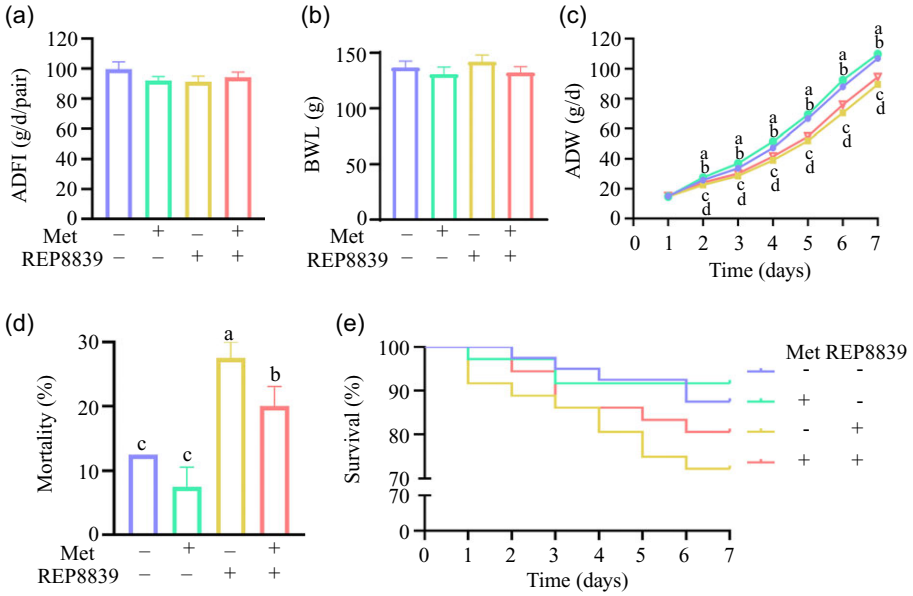

Fig. 7. Effects of methionine (Met) on the productive performance of breeding pigeons and squab growth after REP8839 injection. (a) Average daily feed intake (ADFI) of breeding pigeons. (b) Body weight loss (BWL) of breeding pigeons. (c) Average daily weight (ADW) of squabs over 7 d during lactation. (d) Mortality of squabs. (e) The survival rate of the squabs over 7 d during lactation. The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0{\cdot}05$, n 10).

intake of sows must be maximised during the lactation period because nutrition can directly affect milk production[30]. This opposite result may be because breeding pigeons need more Met to meet their requirements for milk synthesis and baby squab growth in the lactation period.

Pigeon squabs have a greater growth rate in poultry[1], and the growth status of squabs is highly dependent on the nutritional status of their parents. A lower intake of amino acids than the amount required by pigs limits milk production and the growth of offspring[31]. Our results indicated that Met supplementation increased the ADG of squabs and decreased the mortality rate. Moreover, the survival rate after hatching tended to increase in response to Met supplementation. This was consistent with the findings of Zhong *et al.*[13], who reported that Met or methionyl-Met supplementation decreased the mortality rate and increased the ADG of squabs. In addition, Met supplementation improved the ADG of calves during pregnancy. These results suggest that sufficient Met intake is necessary to meet the nutritional needs of parent pigeons and has a positive effect on squab growth.

The crop of pigeon both stores food and produces a 'milk-like substance' during the lactation period. Crop development reflects crop milk synthesis ability to a certain extent. In mammals, the weight and structure of the mammary gland gradually change from the late incubation period to the lactation period[32]. In pigeons, the weight and thickness of the crop increased during the lactation period (7 and 14 d after hatching) after the breeding pigeons were given Met. As reported by Chen *et al.*[16], Met supplementation increased the weight and thickness of crops on the 3rd day of the lactation period in breeding pigeons. This could explain why nutrients might play a key role during the incubation period, contributing to the improvement of crop tissue morphology during the lactation period. Moreover, we found that the weight and thickness of the crops were greater on day 7 than day 14 during the lactation period. Hu *et al.*[3] demonstrated that the weight and thickness of crops peaked in the first week of the lactation period. It is reasonable that crop milk gradually decreases after the first week of the lactation period[1].

The crop of breeding pigeons has the ability to produce milk, which is determined by the number of secreting cells. PCNA is an endogenous nuclear protein that plays an important role in the localisation and quantification of proliferating cells in tissues[33]. Keratin 19 (KRT19) is an intermediate I keratin that can maintain the integrity of epithelial cells and form the cytoskeleton[34,35]. Our results showed that PCNA and CRT19 were highly expressed after breeding pigeons were supplemented with Met. A higher expression of PCNA could contribute to epithelial cell proliferation, and the number of epithelial cells correlates with milk yield in mammals[36], although the crop milk yield of breeding pigeons cannot be determined. Zhu *et al.*[37] demonstrated that KRT19 was involved in the keratinisation of epithelial cells in pigeon crops and facilitated the thickening of epithelial payers with obvious lateral fissures and nail structures. KRT19 may be involved in the process of epithelial differentiation[38]. Taken together, these results suggest that Met supplementation promotes thickening of the crop epithelium with rapid proliferation and keratinisation of epithelial cells in breeding pigeons.

The nutrients in milk, such as proteins, are enriched in crops with massive proliferation of epithelial cells[39]. The main component of milk protein is casein, which is the best-characterised protein and consists of various subunits, including αS1-casein, αS2-casein and β-casein[40]. The present study showed that Met supplementation improved the protein secretion of αS1-casein, αS2-casein and β-casein compared with

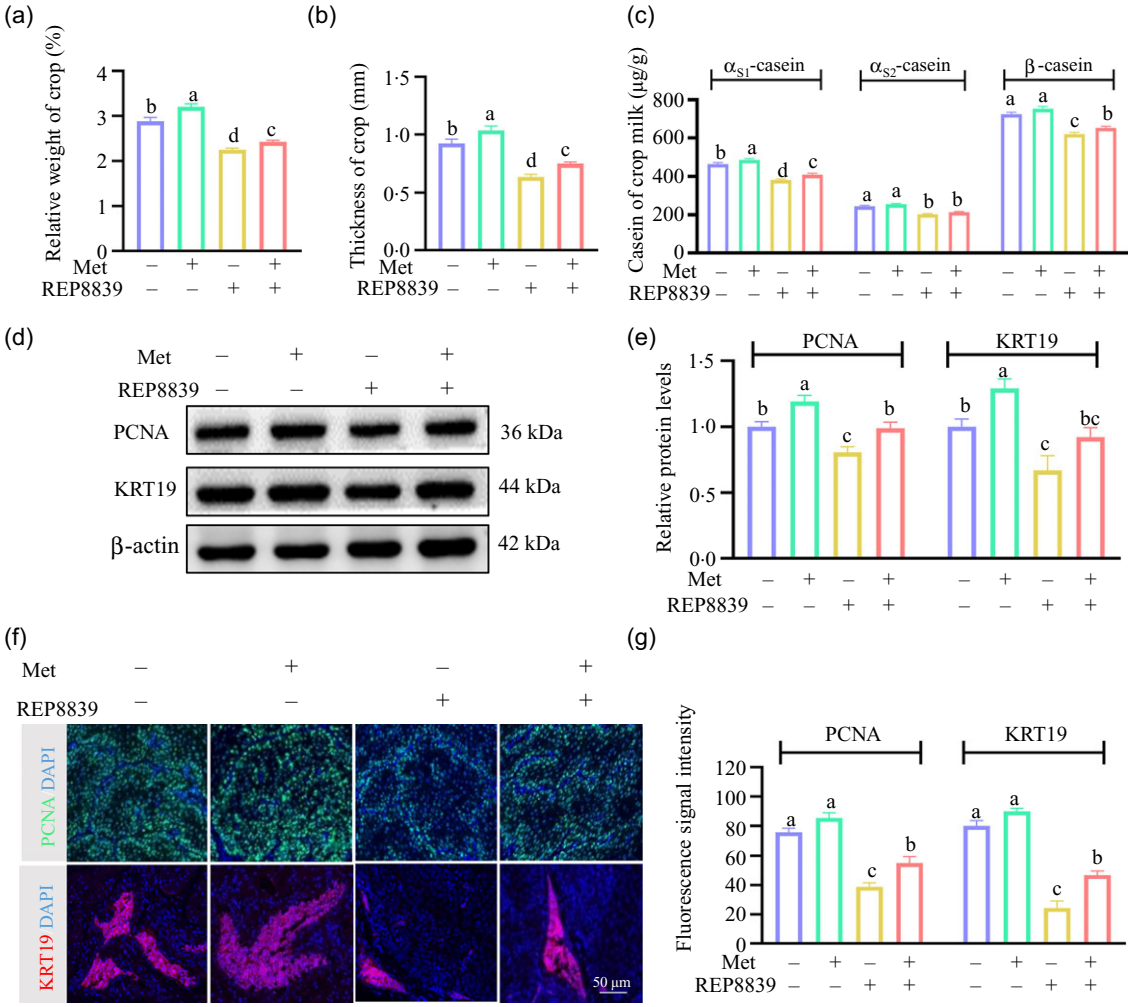

Fig. 8. Effects of methionine (Met) on crop development, crop milk protein levels and crop epithelial cell proliferation and differentiation in breeding pigeons after REP8839 injection. (a) Relative weight of the crop ($n=10$). (b) Thickness of the crop ($n=10$). (c) Levels of α_{S1}-casein, α_{S2}-casein and β-casein in crop milk ($n=10$). (d) Western blot analysis of proliferating cell nuclear antigen (PCNA) and Keratin 19 (KRT19) ($n=3$). (e) Densitometric quantification of PCNA and KRT19. (f) Representative images of immunofluorescence staining for PCNA and KRT19 (scale bar = 50 μm). (g) Fluorescence intensity of PCNA and KRT19 ($n=3$). The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P<0·05$).

that in the Met-deficient group. This finding was consistent with a previous report[13] reporting that Met or methionyl-Met supplementation improved the casein content of crop milk in breeding pigeons. High-quality milk protein is enriched with immunoglobulins and biologically active substances needed for the growth of squabs[41], which supports the positive results of squab growth obtained in this study.

Crop milk protein synthesis depends on crop epithelial cell proliferation, which is regulated by different signalling molecules. JAK2 and STAT5 contribute to the proliferation, differentiation and survival of mammary gland epithelial cells[42]. Amino acid deficiency can cause a reduction in casein transcription by inhibiting the JAK2/STAT5 signalling pathway[43]. Chen *et al.*[16] reported that crop milk protein synthesis was controlled by the JAK2/STAT5 signalling pathway when breeding pigeons were fed Met. Yang *et al.*[8] also indicated that the casein protein level in the bovine mammary gland was increased by activating the JAK2/STAT5 signalling pathway via methionyl-Met supplementation. In agreement with these findings, our results also proved that the JAK2/STAT5 signalling pathway could be activated by Met supplementation in breeding pigeons. The activated signalling pathway may be due to the increase in MetRS protein expression, which we will discuss later. In addition, compared with Met deficiency, Met supplementation decreased SOCS3 protein expression. SOCS3 is a negative regulator of cytokine receptor signalling via the JAK2/STAT5 signalling pathway[44], and decreased SOCS3 expression is required for milk protein synthesis and cell proliferation in mammary epithelial cells[45]. Undoubtedly, the activation of the JAK2/STAT5 signalling pathway promotes an increase in casein for milk protein[14]. Our results showed that protein expression in casein and WAP was increased by Met supplementation. αS1-casein protein expression was stimulated in Met-deficient mice by methionyl-Met supplementation[46]. The casein and WAP of crops are known to be involved in the successful onset of lactation and are responsible for high milk protein content. As reported by Zhou *et al.*[47], the upregulation of casein by Met supplementation can act as a proxy for determining milk protein

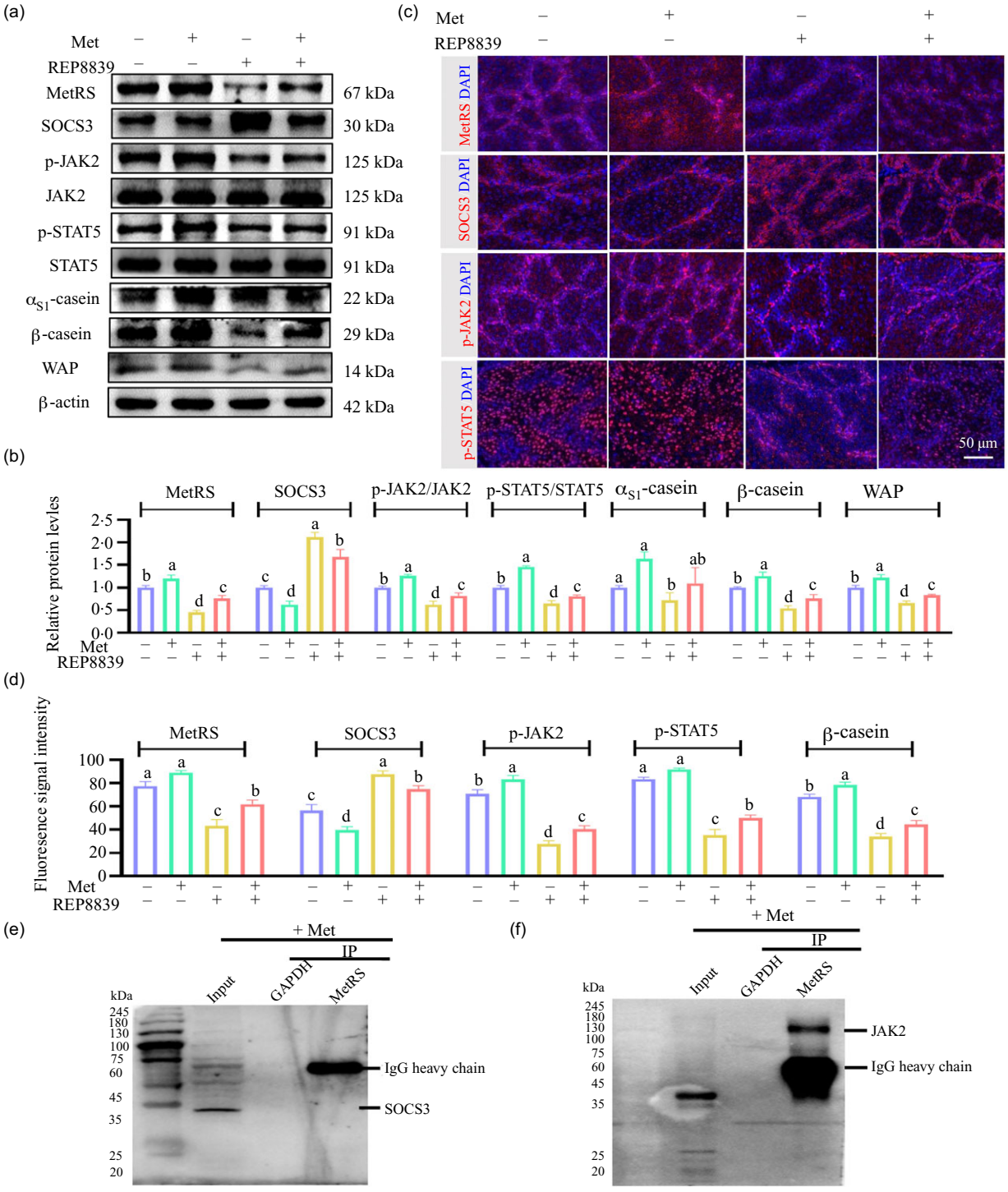

Fig. 9. Effects of methionine (Met) on methionyl-tRNA synthetase (MetRS), the JAK2/STAT5 signalling pathway and crop milk protein synthesis in breeding pigeons after REP8839 injection. (a) Western blot analyses of MetRS, suppressor of cytokine signalling 3 (SOCS3), phosphorylated janus kinase 2 (p-JAK2), JAK2, phosphorylated signal transducer and activator of transcription 5 (p-STAT5), STAT5, αs1-casein, β-casein and whey acidic protein (WAP). (b) Densitometric quantification of MetRS, SOCS3, p-JAK2/JAK2, p-STAT5/STAT5, αs1-casein, β-casein and WAP. (c) Representative images of immunofluorescence staining for MetRS, SOCS3, p-JAK2 and p-STAT5 (scale bar = 50 μm). (d) Fluorescence intensity of MetRS, SOCS3, p-JAK2, p-STAT5 and β-casein. (e) Identification of the interaction between MetRS and SOCS3 by co-immunoprecipitation (co-IP). (f) Identification of the interaction between MetRS and JAK2 by co-IP. The results are presented as the mean ± SEM. Bars with different lowercase letters indicate significant differences ($P < 0{\cdot}05$, n 3).

synthesis in bovine mammary gland cells. These results suggest that Met supplementation improves milk protein synthesis by activating the JAK2/STAT5 signalling pathway.

The function of MetRS in Met-induced milk protein synthesis via the JAK2/STAT5 signalling pathway was further explored in this study. We hypothesised that MetRS inhibits milk protein

https://doi.org/10.1017/S0007114524001181 Published online by Cambridge University Press

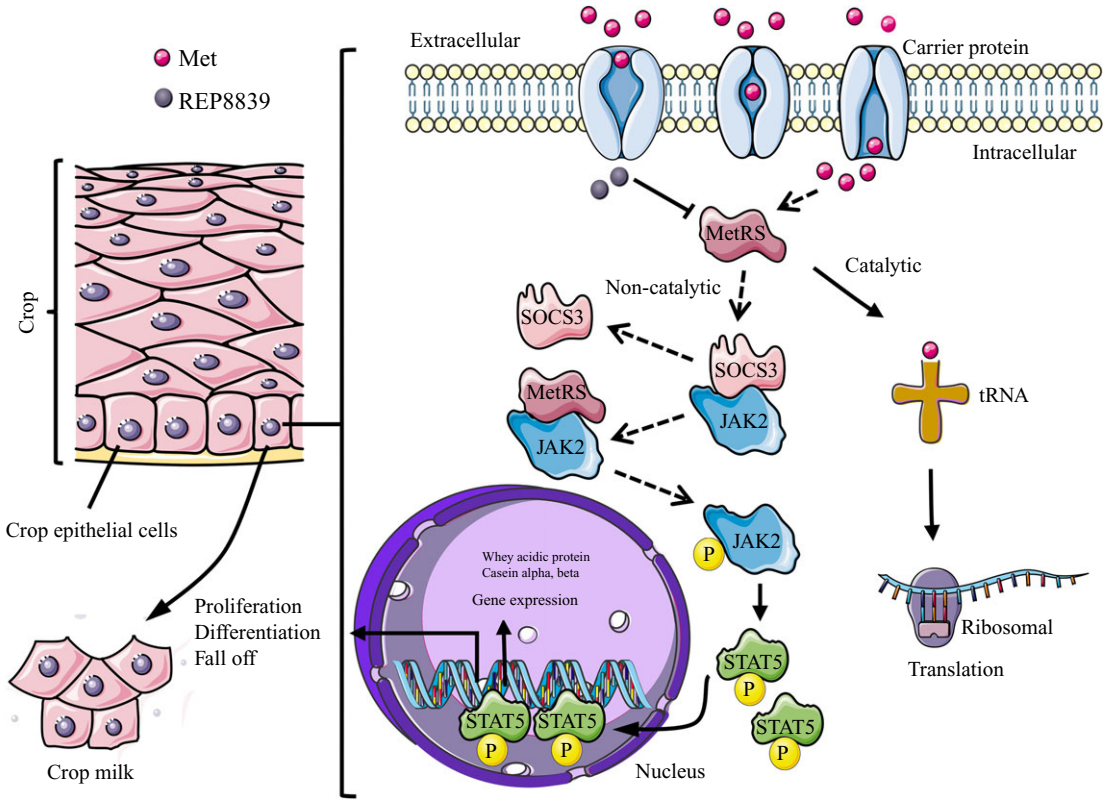

Fig. 10. A schematic diagram summarising the mechanisms by which methionyl-tRNA synthetase (MetRS) is involved in methionine (Met)-induced crop milk protein synthesis in breeding pigeons is shown. Met stimulates the interaction of MetRS with janus kinase 2 (JAK2), activating the JAK2/signal transducer and activator of transcription 5 (STAT5) signalling pathway and subsequently promoting the proliferation and differentiation of crop epithelial cells, leading to crop milk protein synthesis.

synthesis by suppressing the JAK2/STAT5 signalling pathway. REP8839 is designated an inhibitor of aminoacyl-tRNA[48]. It has been reported that REP8839 can specifically disturb protein synthesis during macromolecular synthesis[49]. We first determined the optimal dose of the inhibitor by injecting different concentrations of REP8839 into lactating breeding pigeons to inhibit MetRS-mediated Met-induced milk protein synthesis via the JAK2/STAT5 signalling pathway. The results confirmed that MetRS protein expression decreased, SOCO3 protein expression increased and the p-JAK2/JAK2 and p-STAT5/STAT5 ratios decreased at 1·0 mg/kg BW and 1·5 mg/kg BW, respectively, after injection of REP8839, resulting in a decrease in crop milk protein synthesis, an increase in mortality rate and growth retardation in squabs; moreover, REP8839 impaired crop development, and crop epithelial cells were unable to proliferate and differentiate in breeding pigeons. Therefore, we selected 1·0 mg/kg of REP8839 as the optimal dose for Experiment 3.

The MetRS inhibitor (REP8839) competes with Met[50]. A higher dose of Met may inhibit the optimal binding of REP8839 to MetRS. We therefore injected 1·0 mg/kg REP8839 in combination with Met to determine the effects of MetRS on milk protein synthesis. These results were consistent with those obtained in Experiment 2; REP8839 inhibited squab growth and increased the mortality rate without Met supplementation. This poor squab growth could be explained by the crop development of the breeding pigeons. REP8839 inhibited crop development and the expression of the PCNA and KRT19 proteins, suggesting that the inhibition of crop development negatively affects the proliferation and keratinisation of crop epithelial cells, leading to a decrease in the αS1-casein and β-casein levels. Similarly, knockdown of SerRS decreased cell proliferation and β-casein expression in bovine mammary epithelial cells[6]. A decrease in β-casein in mammary epithelial cells was also observed upon knockdown of leucyl-tRNA synthetase[51]. As expected, Met supplementation compensated for the growth and mortality rate of squabs after the injection of REP8839. This difference may be due to the increase in the protein expression of PCNA and KRT19, which accelerate the proliferation and keratinisation of crop epithelial cells and promote milk protein synthesis in breeding pigeons.

We next determined whether MetRS can be stimulated by both REP8839 and Met to subsequently control the JAK2/STAT5 signalling pathway. First, REP8839 interfered with the protein expression of MetRS, increased SOCOS3 protein expression, inhibited the activation of the JAK2 and STAT5 signalling pathway and decreased the protein expression of β-casein without Met supplementation. However, Met supplementation increased MetRS expression and decreased SOCS3 expression, activated the JAK2/STAT5 signalling pathway and increased β-casein protein expression after REP8839 injection. These findings suggested that MetRS can respond to stimulation by Met and REP8839. Similarly, mammary epithelial cells treated with Met exhibited decreased SOCS3 protein expression and increased STAT5 and β-casein protein expression. Moreover,

SOCS3 is an inhibitor of the JAK2/STAT5 signalling pathway and can regulate the protein expression of STAT5 and β-casein through knockdown and overexpression[45]. Dai *et al.*[20] also reported that Met reactivated the protein expression of SerRS and promoted the protein abundance of β-casein after SerRS was knocked down in mammary epithelial cells. These results may imply that sufficient Met rescues the inhibitory effect of REP8839 on MetRS expression and activates the JAK2/STAT5 signalling pathway, thus contributing to milk protein synthesis.

Moreover, the Co-IP results verified that MetRS and JAK2 interact. These results confirmed that Met activates MetRS and interacts with JAK2 to form protein complexes, thereby promoting the proliferation and differentiation of crop epithelial cells and increasing the protein synthesis of crop milk.

Conclusion

In conclusion, the current study revealed that Met supplementation improved crop milk protein synthesis and squab growth. The underlying mechanism may involve the stimulation of MetRS by Met, which activates the JAK2/STAT5 signalling pathway via interaction with JAK2 and MetRS, ultimately resulting in crop protein synthesis through the proliferation and differentiation of crop epithelial cells in breeding pigeons (Fig. 10). These findings could expand our understanding of the mechanisms by which Met regulates milk protein synthesis in animals and provide insight into maternal–infant nutrition in humans.

Acknowledgements

The authors thank all of the participants, coordinators and administrators for their support and help during the research.

Financial support

This work was supported by the National Natural Science Foundation of China (32172746), the Guangdong Chimelong Philanthropic Foundation (CLPF2021007Z) and the Natural Science Foundation of Guangdong Province, China (2019B1515210001).

Authorship

C. G. and C. Z. designed the research; C. Z. and H. Z. conducted the research; C. Z. analysed data; P. L. wrote the manuscript draft; F. L., X. W. and C. G. supervised the trial; P. L., H. Y., X. W. and C. G. contributed to manuscript revision. All authors read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114524001181.

References

1. Sales J & Janssens G (2003) Nutrition of the domestic pigeon (Columba livia domestica). *World's Poult Sci J* **59**, 221–232.
2. Xie W, Fu Z, Pan N, *et al.* (2019) Leucine promotes the growth of squabs by increasing crop milk protein synthesis through the TOR signaling pathway in the domestic pigeon (Columba livia). *Poult Sci* **98**, 5514–5524.
3. Hu XC, Gao CQ, Wang XH, *et al.* (2016) Crop milk protein is synthesized following activation of the IRS1/Akt/TOR signaling pathway in the domestic pigeon (Columba livia). *Br Poult Sci* **2016**, 855–862.
4. Oftedal OT (2012) The evolution of milk secretion and its ancient origins. *Animal* **6**, 355–368.
5. Luo C, Zhao S, Zhang M, *et al.* (2018) SESN2 negatively regulates cell proliferation and casein synthesis by inhibition the amino acid-mediated mTORC1 pathway in cow mammary epithelial cells. *Sci Rep* **8**, 3912.
6. Dai W, White R, Liu J, *et al.* (2018) Seryl-tRNA synthetase-mediated essential amino acids regulate β-casein synthesis via cell proliferation and mammalian target of rapamycin (mTOR) signaling pathway in bovine mammary epithelial cells. *J Dairy Sci* **101**, 10456–10468.
7. Bionaz M & Loor JJ (2011) Gene networks driving bovine mammary protein synthesis during the lactation cycle. *Bioinform. Bio. Insights* **5**, 83–98.
8. Yang JX, Wang CH, Xu QB, *et al.* (2015) Methionyl-Methionine promotes α-s1 casein synthesis in bovine mammary gland explants by enhancing intracellular substrate availability and activating JAK2-STAT5 and mTOR-mediated signaling pathways. *J Nutr* **145**, 1748–1753.
9. Brosnan JT, Brosnan ME, Bertolo RF, *et al.* (2007) Methionine: a metabolically unique amino acid. *Livest Sci* **11**, 2–7.
10. Wang C, Liu H, Wang Y, *et al.* (2010) Effects of dietary supplementation of methionine and lysine on milk production and nitrogen utilization in dairy cows. *J Dairy Sci* **93**, 3661–3670.
11. Kim JE & Lee HG (2021) Amino acids supplementation for the milk and milk protein production of dairy cows. *Animals* **11**, 2118.
12. McFadden J, Girard C, Tao S, *et al.* (2020) Symposium review: one-carbon metabolism and methyl donor nutrition in the dairy cow. *J Dairy Sci* **103**, 5668–5683.
13. Zhong C, Zhang Y, Pan N, *et al.* (2022) Dietary DL-methionine replacement by a novel DL-methionyl-DL-methionine dipeptide increases milk protein synthesis in breeding pigeons and improves the growth performance of squabs. *Anim Feed Sci Tech* **292**, 115420.
14. Nan X, Bu D, Li X, *et al.* (2014) Ratio of lysine to methionine alters expression of genes involved in milk protein transcription and translation and mTOR phosphorylation in bovine mammary cells. *Physiol Genomics* **46**, 268–275.
15. Hou X, Jiang M, Zhou J, *et al.* (2020) Examination of methionine stimulation of gene expression in dairy cow mammary epithelial cells using RNA-sequencing. *J Dairy Res* **87**, 226–231.
16. Chen M, Pan N, Wang X, *et al.* (2020) Methionine promotes crop milk protein synthesis through the JAK2-STAT5 signaling during lactation of domestic pigeons (Columba livia). *Food Funct* **11**, 10786–10798.

British Journal of Nutrition

17. Yao P & Fox PL (2020) Aminoacyl-tRNA synthetases in cell signaling. In *The Enzymes*, pp. 243–275 [LR Pouplana and LS Kaguni, editors]. Cambridge: Academic Press.
18. Yu YC, Han JM & Kim S (2021) Aminoacyl-tRNA synthetases and amino acid signaling. *Biochim Biophys Acta Mol Cell Res* **1868**, 118889.
19. Luo C, Qi H, Huang X, *et al.* (2019) GlyRS is a new mediator of amino acid-induced milk synthesis in bovine mammary epithelial cells. *J Cell Physiol* **234**, 2973–2983.
20. Dai W, Zhao F, Liu J, *et al.* (2020) Seryl-tRNA synthetase is involved in methionine stimulation of β-casein synthesis in bovine mammary epithelial cells. *Br J Nutr* **123**, 489–498.
21. Lin X, Li S, Zou Y, *et al.* (2018) Lysine stimulates protein synthesis by promoting the expression of $ATB^{0,+}$ and activating the mTOR pathway in bovine mammary epithelial cells. *J Nutr* **148**, 1426–1433.
22. Zhang Y, Li F, Lu Z, *et al.* (2023) L-Malic acid facilitates stem cell-driven intestinal epithelial renewal through the amplification of β-catenin signaling by targeting Frizzled7 in chicks. *J Agric Food Chem* **71**, 13079–13091.
23. Zhong C, Tong D, Zhang Y, *et al.* (2022) DL-methionine and DL-methionyl-DL-methionine increase intestinal development and activate Wnt/β-catenin signaling activity in domestic pigeons (Columba livia). *Poult Sci* **101**, 101644.
24. Jariyahatthakij P, Chomtee B, Poeikhampha T, *et al.* (2018) Effects of adding methionine in low-protein diet and subsequently fed low-energy diet on productive performance, blood chemical profile, and lipid metabolism-related gene expression of broiler chickens. *Poult Sci* **97**, 2021–2033.
25. Liu Y, Wang D, Zhao L, *et al.* (2022) Effect of methionine deficiency on the growth performance, serum amino acids concentrations, gut microbiota and subsequent laying performance of layer chicks. *Front Vet Sci* **9**, 878107.
26. Wu Y, Tang J, Cao J, *et al.* (2021) Effect of dietary L-methionine supplementation on growth performance, carcass traits, and plasma parameters of starter pekin ducks at different dietary energy levels. *Animals* **11**, 144.
27. Fagundes NS, Milfort MC, Williams SM, *et al.* (2020) Dietary methionine level alters growth, digestibility, and gene expression of amino acid transporters in meat-type chickens. *Poult Sci* **99**, 67–75.
28. Wathes D, Fenwick M, Cheng Z, *et al.* (2007) Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. *Theriogenology* **68**, S232–41.
29. Reda FM, Swelum AA, Hussein EO, *et al.* (2020) Effects of varying dietary DL-methionine levels on productive and reproductive performance, egg quality, and blood biochemical parameters of quail breeders. *Animals* **10**, 1839.
30. Gauthier R, Largouët C, Gaillard C, *et al.* (2019) Dynamic modeling of nutrient use and individual requirements of lactating sows. *J Anim Sci* **97**, 2822–2836.
31. Guan X, Pettigrew J, Ku P, *et al.* (2004) Dietary protein concentration affects plasma arteriovenous difference of amino acids across the porcine mammary gland. *J Anim Sci* **82**, 2953–2963.
32. Knight C & Peaker M (1982) Development of the mammary gland. *Reprod* **65**, 521–536.
33. Nguyen HD & Bielinsky AK (2010) HDM2 ERKs PCNA. *J Cell Biol* **190**, 487.
34. Moll R, Divo M & Langbein L (2008) The human keratins: biology and pathology. *Histochem Cell Biol* **129**, 705–733.
35. Li X, Dong X, Xue Y, *et al.* (2014) Increased expression levels of E-cadherin, cytokeratin 18 and 19 observed in preeclampsia were not correlated with disease severity. *Placenta* **35**, 625–631.
36. Boutinaud M, Guinard-Flament J & Jammes H (2004) The number and activity of mammary epithelial cells, determining factors for milk production. *Reprod Nutr Dev* **44**, 499–508.
37. Zhu J, Xie P, Zheng M, *et al.* (2023) Dynamic changes in protein concentrations of keratins in crop milk and related gene expression in pigeon crops during different incubation and chick rearing stages. *Br Poult Sci* **64**, 100–109.
38. Yang J, Xiong L, Wang R, *et al.* (2015) *I*n vitro expression of cytokeratin 18, 19 and tube formation of adipose-derived stem cells induced by the breast epithelial cell line HBL-100. *J Cell Mol Med* **19**, 2827–2831.
39. Gillespie MJ, Haring VR, McColl KA, *et al.* (2011) Histological and global gene expression analysis of the 'lactating' pigeon crop. *BMC Genom* **12**, 452.
40. Bhat MY, Dar TA & Singh LR (2016) Casein proteins: structural and functional aspects. In *Milk Proteins-From Structure to Biological Properties and Health Aspects*, pp. 64187 I Gigli, editor. Rijeka: InTech.
41. Jin C, He Y, Jiang S, *et al.* (2023) Chemical composition of pigeon crop milk and factors affecting its production: a review. *Poult Sci* **102**, 102681.
42. Tian M, Qi Y, Zhang X, *et al.* (2020) Regulation of the JAK2-STAT5 pathway by signaling molecules in the mammary gland. *Front Cell Dev Biol* **8**, 604896.
43. Zhang M, Zhao S, Wang S, *et al.* (2018) D-Glucose and amino acid deficiency inhibits casein synthesis through JAK2/STAT5 and AMPK/mTOR signaling pathways in mammary epithelial cells of dairy cows. *J Dairy Sci* **101**, 1737–1746.
44. Kim WS, Kim MJ, Kim DO, *et al.* (2017) Suppressor of cytokine signaling 2 negatively regulates NK cell differentiation by inhibiting JAK2 activity. *Sci Rep* **7**, 46153.
45. Huang YL, Zhao F, Luo CC, *et al.* (2013) SOCS3-mediated blockade reveals major contribution of JAK2/STAT5 signaling pathway to lactation and proliferation of dairy cow mammary epithelial cells *i*n vitro. *Molecules* **18**, 12987–13002.
46. Chen Q, Zhao F, Ren Y, *et al.* (2020) Parenterally delivered methionyl-methionine dipeptide during pregnancy enhances mammogenesis and lactation performance over free methionine by activating PI3K-AKT signaling in methionine-deficient mice. *J Nutr* **150**, 1186–1195.
47. Zhou J, Yue S, Xue B, *et al.* (2021) Enhanced supply of methionine regulates protein synthesis in bovine mammary epithelial cells under hyperthermia condition. *J Anim Sci Technol* **63**, 1126–1141.
48. Ochsner UA, Sun X, Jarvis T, *et al.* (2007) Aminoacyl-tRNA synthetases: essential and still promising targets for new anti-infective agents. *Expert opin Invest Drugs* **16**, 573–593.
49. Ochsner UA, Young CL, Stone KC, *et al.* (2005) Mode of action and biochemical characterization of REP8839, a novel inhibitor of methionyl-tRNA synthetase. *Antimicrob Agents Chemother* **49**, 4253–4262.
50. Green LS, Bullard JM, Ribble W, *et al.* (2009) Inhibition of methionyl-tRNA synthetase by REP8839 and effects of resistance mutations on enzyme activity. *Antimicrob Agents Chemother* **53**, 86–94.
51. Wang L, Lin Y, Bian Y, *et al.* (2014) Leucyl-tRNA synthetase regulates lactation and cell proliferation via mTOR signaling in dairy cow mammary epithelial cells. *Int J Mol Sci* **15**, 5952–5969.