Postnatal weight gain induced by overfeeding pups and maternal high-fat diet during the lactation period modulates glucose metabolism and the production of pancreatic and gastrointestinal peptides

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Abbreviations

PND, postnatal day; GLP-1, glucagon-like peptide 1, GIP: glucose-dependent insulinotropic polypeptide; GOAT, ghrelin-O-acyltransferase; Pdx-1: pancreas duodenum homeobox-1; Ghrl, ghrelin, Ins, insulin; Gcg, glucagon; PUFA, polyunsaturated fatty acids.

Key words

neonatal weight gain; maternal high-fat diet; insulin; incretin; ghrelin.

Abstract

The impact of rapid weight gain on glucose metabolism during the early postnatal period remains unclear. We investigated the influence of rapid weight gain under different nutritional conditions on glucose metabolism, focusing on the production of pancreatic and gastric peptides. On postnatal day (PND) 2, C57BL/6N pups were divided into three groups: control (C) pups whose dams were fed a control diet (10 %kcal fat) and nursed 10 pups each; maternal high-fat diet (HFD) pups whose dams were fed an HFD (45 %kcal fat) and nursed 10 pups each; and overfeeding (OF) pups whose dams were fed the control diet and nursed 4 pups each. Data were collected on PND 7, 14 and 21. The body weight gains of the HFD and OF pups were 1.2 times higher than that of the C pups. On PND 14, the HFD pups had higher blood glucose levels, but there were no significant differences in serum insulin levels between the HFD and C pups. The OF pups had higher blood glucose and serum insulin levels than that of the C pups. Insulin resistance was found in the HFD and OF pups. On PND 14, the content of incretins in the jejunum was increased in the OF pups, and acyl ghrelin in the stomach was upregulated in the HFD and OF pups. These results suggest that neonatal weight gain induced by overfeeding pups and maternal high-fat diet during the early postnatal period modulates the insulin sensitivity and the production of pancreatic and gastrointestinal peptides.

1. Introduction

The early postnatal period has been suggested to be a crucial window for programming of glucose metabolism that may influence later life. Rapid postnatal weight gain may cause impaired glucose tolerance and insulin sensitivity [6, 35] in childhood, and thus represents a potential risk factor for Type 2 diabetes later in life. Rapid postnatal weight gain can be induced by maternal high fat diet [39] or overfeeding [23] during early postnatal period.

Gastrointestinal and pancreatic peptides play important roles in glucose and energy balance. Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are synthesized by the endocrine cells in the small intestine [30]. The biological action of GLP-1 [16, 26, 29] and GIP [10], which stimulate glucose-dependent insulin secretion, has been well established by previous studies. In addition, each peptide is secreted in response to ingestion of nutrients, especially dietary carbohydrates and fat [2]. Ghrelin is produced predominantly by the stomach and stimulates growth hormone secretion, appetite, and fat accumulation [25, 41]. Recent studies revealed an essential function of ghrelin in maintaining glucose homeostasis [27, 28]. Ghrelin, which contains 28 amino acids, is *N*-octanoylated at Ser3 by ghrelin-*O*-acyltransferase (GOAT) [17, 43], a unique modification that is necessary for ghrelin's activity. Ghrelin acylation can be influenced by changing the fatty-acid composition of the diet [24]. Although these peptides are important for maintaining glucose homeostasis, we know relatively little about their production during the neonatal period.

Numerous recent studies have demonstrated the benefits of breastfeeding [1, 13, 33]. However, due to

62 modern changes in diet, the fat composition of the maternal diet during lactation is high not only in
63 western societies but also in Asia, new mothers consume far more fats and carbohydrates than they need
64 [9]. To date, little is known about the effects of maternal diet on breast milk which directly change the
65 neonatal growth and body composition. Neonatal rapid growth and childhood obesity are associated with
66 the risk of later excessive weight gain and obesity [34, 36, 38]. In this study, using two different postnatal
67 excessive weight gain mouse models (maternal high-fat diet and overfeeding), we investigated metabolic
68 abnormalities related to childhood obesity and changes in gastrointestinal and pancreatic peptide
69 synthesis. Moreover, we assessed in these animals how the composition of breast milk changes in
70 response to maternal diet, and how these changes influence the offspring.
71
72
73 **2. Materials and methods**
74 **2.1. Animals**
75 C57BL/6N mice on day 15 of pregnancy were purchased from SLC Japan (Shizuoka, Japan). During
76 pregnancy, the mice were housed individually under standardized environmental conditions (temperature
77 of 24–25°C, artificial lighting 0700–1900 h). Tap water and standard laboratory chow (CLEA Rodent
78 Diet CE-2, Osaka, Japan) were freely available. All animal procedures were conducted in compliance
79 with protocols approved by Japanese Physiological Society's guidelines for animal care, and were in
80 accordance with the Animal Ethics Committee of National Cerebral and Cardiovascular Center Research

Institute and Mie University.

2.2. Experimental protocol

Experimental design was shown in Fig. 1A. Dams were allowed to deliver spontaneously, and the day of birth was defined as postnatal day 0 (PND 0). Only dams with litter sizes between 6 and 9 were used in this study. Seventy-six pups were randomly distributed into three groups on PND 2, as follows: control pups (C pups, n = 30), whose dams were fed a control diet (3.85 kcal/g with 10% of total calories as fat consisting of soybean oil [5.6%] and lard [4.4%], and 20% as protein; formula D12450B, Research Diets Inc., New Brunswick, NJ, USA) and nursed 10 pups each; maternal high-fat diet pups (HFD pups, n = 30), whose dams were fed an HFD (4.73 kcal/g with 45% of total calories as fat consisting of soybean oil [5.6%] and lard [39.4%], and 20% as protein; formula D12451, Research Diets Inc.) and nursed 10 pups each; and overfeeding pups (OF pups, n = 16), whose dams were fed the control diet and nursed 4 pups each. The fatty-acid compositions of each diet were analyzed by gas chromatography, as described above, and are shown in Table 1. The sex ratios of pups were adjusted to almost 1:1. The C group included 15 males and 15 females; the HFD group included 15 males and 15 females; and the OF group included 10 males and 6 females. Pups were raised with foster mothers during nursing until PND 21. The dams and pups were weighed on PND 2, 7, 14, and 21. Measurements of body length were performed dorsally on pups from the tip of the nose to the base of the tail on PND 14 and 21. The food intakes of the dams were recorded weekly. Measurements of body length were performed dorsally on pups from the tip of the nose

100 to the base of the tail on PND 14 and 21. Body weight gain was calculated as the difference between PND
101 2 and PND 14. Fat tissue weight was calculated as the sum of the subcutaneous fat weight and visceral fat
102 weight of pups. Two dams and their pups from each group were euthanatized on PND 14, and the others
103 were euthanatized on PND 21 between 1000 h and 1200 h under ad libitum feeding conditions to collect
104 blood and tissues of the stomach, jejunum, ileum, and pancreas. On PND 14, stomach milk contents were
105 weighed to assess pup milk intake. All samples were stored immediately at -80°C until analysis.
106
107 **2.3. Assessment of insulin sensitivity and beta-cell function in the pups**
108 On PND 14 and 21, blood was obtained from the fasted pups after the separation from their dams for 4 h
109 to measure blood glucose and serum insulin levels. Each pancreas was also removed to assess the insulin
110 content. The homeostasis model assessment (HOMA) is a method used quantify insulin resistance (-IR)
111 and beta-cell function (-beta). HOMA-IR was calculated using the following formula: fasting insulin
112 (μIU/ml) × fasting glucose (mg/dl) / 405. HOMA-beta was calculated according the formula: [360 ×
113 fasting insulin (μIU/ml)] / [fasting glucose (mg/dl) – 63]. The conversion factor for insulin was 1 ng/ml =
114 26 μIU/ml.
115
116 **2.4. Measurements of blood glucose and peptides**
117 Blood glucose concentrations were measured using the One Touch Ultra Blood Glucose Monitoring
118 system (LIFESCAN, Milpitas, CA, USA). Blood samples were collected from the carotid artery, and sera

were obtained after centrifugation. Serum insulin level and pancreas insulin content were measured using an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Tissue contents of GLP-1 and GIP were measured using the GLP-1 ELISA Kit and the GIP (Active) ELISA Kit, respectively (Wako, Osaka, Japan).

2.5. Purification of stomach, jejunum, ileum and pancreas tissues

Fresh glandular stomach tissue was divided into anterior- and posterior-wall sections, the first for measurement of ghrelin content and the latter for gene-expression analysis. Samples (10–20 mg) of jejunum and ileum were removed. To measure peptide contents, the tissues were quickly diced, frozen, and stored. Each tissue sample was boiled for 10 min in 1 ml of water to inactivate intrinsic proteases. The solution was adjusted to 1 M acetic acid after cooling, and the tissue was homogenized using a TissueLyser (QIAGEN, Hilden, Germany). After centrifugation, the supernatant was lyophilized and subjected to ghrelin RIA [20] and ELISAs for GLP-1 and GIP. For gene-expression analysis, each tissue was stored in 1–1.5 ml of RNAlater (Ambion, Austin, TX, USA). Pancreas tissues from the tail end to the head attached to the duodenum were removed rapidly. After weighed, each tissue was homogenized in 1 ml of ice cold acid ethanol (0.18 M hydrochloric acid in 70% ethanol) [3] with TissueLyser. After centrifugation, the supernatant was lyophilized and subjected to ELISA for insulin.

2.6. Radioimmunoassays for Ghrelin

Stomach levels of total and acylated ghrelin were measured using two specific radioimmunoassay (RIA) systems, as previously described [20, 21]. We have established two ghrelin-specific RIAs: N-RIA recognizes the N-terminal, octanoyl-modified portion of the peptide, whereas C-RIA recognizes the C-terminal portion. Thus, the value determined by N-RIA specifically measures active ghrelin, whereas the value determined by C-RIA gives the total ghrelin immunoreactivity, including both active and des-acyl ghrelin.

2.7. Analysis of gene expression by quantitative RT- PCR

Total RNAs were extracted from the various tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Each sample was placed into a 2-ml microcentrifuge tube containing 1 ml of TRIzol reagent, and homogenized using a TissueLyser apparatus. Target genes are listed in Table 2. Quantitative reverse transcription PCR (RT-qPCR) was performed on a LightCycler system (Roche Diagnostics, Mannheim, Germany) using One-Step SYBR PrimeScript RT-PCR Kit II (TaKaRa, Shiga, Japan) with the listed primer sets. C_T values were recorded automatically, and the known starting concentrations of standard cDNAs were used to construct each calibration curve. Target-gene expression was then normalized to 36B4 gene expression. At the end of the PCR, melting-curve analysis was performed to verify product specificity. The predicted length of each product was confirmed by agarose gel electrophoresis.

2.8. Milk composition analysis

157 For milk composition analysis, another set of dams (Control dams, n = 5; HFD dams, n = 5; OF dams,
158 n = 3) was prepared as described above. Each dam was injected intraperitoneally with 4 units of oxytocin
159 (Sigma Aldrich, Louis, MO, USA) in the morning on PND 14, and 15 min later milk was collected into
160 glass capillary tubes (UCHIDA, Tokyo, Japan) using a vacuum pump. Total triglyceride was measured
161 using the Triglyceride E-Test kit (Wako) based on the GPO-DAOS method. Measurements were
162 considered to represent the total fat content of the milk. Total solid was calculated from the weight of
163 dried matter after a 4 h-incubation at 100 ± 2°C in a microtube. The dried matter was subsequently
164 incinerated at 550°C for 12 h to determine ash content. Lactose content was determined using the
165 Lactose/D-Galactose kit (R-Biopharm AG, Darmstadt, Germany), based on measurement of UV
166 absorbance after lactose and galactose hydrolysis. The measurements were considered to represent the
167 carbohydrate content of the milk. Protein content and gross total energy content were calculated as
168 follows: protein (g/100 ml) = total solid – fat – carbohydrate – ash; energy (kcal/100 ml) = protein × 4 +
169 fat × 9 + carbohydrate × 4.
170
171 **2.9. Fatty acid composition analysis**
172 Total fat of milk, pup serum, and experimental diets were extracted by the method of Folch et al [15].
173 Methyl esters of fatty acids were prepared from total fat by the boron trifluoride–methanol method. Fatty
174 acid composition of the esters was analyzed using a Shimadzu model GC-2010 PlusAF gas
175 chromatograph (Kyoto, Japan) fitted with capillary column DB-WAX. The analysis conditions were as

follows: capillary column, 30 m × 0.250 mm i.d.; column temperature from 160 to 220°C (1.5°C/min); injection and detector temperature, 240 and 250°C, respectively; detector, FID; carrier gas, helium; flow rate, 0.04 l/min; split ratio 1:100.

2.10. Statistical analysis

All data are expressed as means ± SE. Comparisons of parameters between groups were made with one-way ANOVA followed by Bonferroni post hoc test or paired t-test. A value of $P < 0.05$ was considered statistically significant. The IBM SPSS Statistics software version 19.0.0 was used for statistical analyses.

3. Results

3.1. Changes in maternal body weight and food intake

There were no significant differences in body weights of the dams among the three groups on PND 2, 7, and 14 (Fig. 1B). The cumulative caloric intake of the HFD dams from PND 2 to 14 was significantly higher than that of the C dams, whereas that of the OF dams was significantly lower than that of the C dams (Fig. 1C).

3.2. Litter growth and blood chemistry

There was no significant difference in the body weights of pups among the three groups on PND 2 (Fig. 2A). Body weights of the HFD and OF pups were higher significantly than those of the C pups on PND 7, 14, and 21. Total body weight gains from PND 2 to 14 were significantly higher in the HFD pups (6.11 ± 0.07 g) and OF pups (6.98 ± 0.44 g) than in the C pups (4.86 ± 0.13 g) ($P < 0.01$), whereas there was no significant difference between the HFD and OF pups. On PND 14, the stomach milk content of the OF pups was approximately 2-fold higher than that of the C and HFD pups (Fig. 2B). There was no significant difference in stomach milk content between the HFD and C pups. Body lengths of the HFD and OF pups were significantly longer than those of the C pups on PND 14 and 21 (Fig. 2C). Fat tissue weights in the C pups (n=10), HFD pups (n=10) and OF pups (n=8) were 0.114 ± 0.002 g, 0.199 ± 0.016 and 0.185 ± 0.006 on PND 14, respectively; 0.172 ± 0.009 g, 0.268 ± 0.019 and 0.237 ± 0.013 on PND 21, respectively. Fat tissue weights were significantly higher in the HFD and OF pups than those in the C pups on PND 14 and PND 21 ($P < 0.01$).

Blood glucose levels were significantly higher in the HFD and OF pups relative to the C pups on PND 14, but there were no significant differences among the three groups on PND 21 (Fig. 2D). Serum insulin levels were significantly higher in the OF pups than in the C pups on PND 14 and 21. By contrast, there were no significant differences in serum insulin levels between the HFD and C pups on PND 14 and 21 (Fig. 2E).

3.3. Gene-expression analysis in the pancreas

There were no significant differences in insulin, proglucagon, or pancreas duodenum homeobox-1 (Pdx-1) mRNA levels between the HFD and C pups on PND 14 and 21. By contrast, on PND 21 the OF pups had significantly higher insulin and Pdx-1 mRNA levels than the C pups. On PND 14 and 21, proglucagon mRNA levels were significantly higher in the OF pups than in the C pups (Fig. 3A–C). Chymotrypsinogen B1 mRNA levels on PND 14 were significantly higher in the HFD and OF pups than in the C pups, but there were no significant differences among the three groups on PND 21 (Fig. 3D).

3.4. Gene expression and peptide content analysis in the small intestine

On PND 14, the mRNA levels of proglucagon and GIP in the jejunum and proglucagon in the ileum were significantly higher in the HFD pups than in the C pups, but on PND 21 the mRNA levels of proglucagon in the ileum were significantly lower in the HFD pups than in the C pups (Fig. 4A–D). The mRNA levels of proglucagon and GIP in the jejunum and GIP in the ileum were significantly higher in the OF pups than in the C pups on PND 14, but on PND 21 no significant differences were observed between the OF and C pups in proglucagon and GIP mRNA levels in the jejunum and ileum.

The content of active GIP peptide in the jejunum was significantly higher in the OF pups than in the C pups (Fig. 4E). Jejunal GLP-1 content also tended to be higher compared to the C pups, but the difference was not significant. There were no significant differences between the HFD and C pups in jejunal content of GIP and GLP-1.

3.5. Gene expression and peptide content analysis of stomach ghrelin

In HFD pups, the acylated ghrelin content and the ratio of acylated ghrelin to total ghrelin were significantly higher than in C pups on PND 14 (Fig. 5A and B). Similarly, on PND 14, the OF pups had significantly higher levels of acylated ghrelin content, total ghrelin content, and ratio of acylated ghrelin to total ghrelin than the C pups (Fig. 5C). On PND 21, however, no significant differences were observed among the three groups in acylated and total ghrelin content. Furthermore, there were no significant differences among the three groups in ghrelin or GOAT mRNA levels PND 14 (Fig. 5D).

3.6. Assessment of insulin sensitivity and beta-cell function in excessive weight gain pups

We evaluated the differences of glucose/insulin metabolism between two excessive weight gain pups by comparing HOMA-IR and HOMA-beta. HOMA-IR levels were significantly higher in the HFD and OF pups than in the C pups On PND 14, but there was no significant change in three groups on PND 21 (Fig. 6A). HOMA-beta on PND 14 showed a significantly higher in the OF pups compared to in the HFD and C pups. Similarly, on PND 21, HOMA-beta in the OF pups was increased, however the change did not reach significance difference between the OF and C pups (Fig. 6B).

Pancreas insulin contents in fasting status did not change in three groups on PND 14. In the HFD pups, the insulin contents had small but significant increase compared with the C pups on PND 21 (Fig. 6C).

3.7. Milk composition analysis

252 The average content of fat, protein, lactose, and energy in milk is shown in Table 3. Fatty-acid
253 composition of milk on PND 14 is shown in Table 4.
254
255 **3.8. Fatty acid composition changes in litter serum.**
256 The composition of fatty acids in litter serum was influenced by the milk (Fig. 7). On PND 14, the
257 compositions of palmitic acid (C16:0), palmitoleic acid (C16:1), and oleic acid (C18:1) were significantly
258 lower in the HFD pups compared than in the C pups, whereas the compositions of stearic acid (C18:0),
259 linoleic acid (C18:2), and arachidonic acid (C20:4) were significantly higher in the HFD pups than in the
260 C pups. There were no significant differences between the HFD and C pups in the content of other fatty
261 acids on PND 14.
262
263
264 **4. Discussion**
265 Early postnatal weight gain is associated with obesity in later childhood and adolescence [8]. In this study,
266 we focused on the influence of nutritional diet on offspring metabolic phenotype, using two different
267 neonatal excessive weight gain models (pup overfeeding [OF] and maternal high-fat diet [HFD]). In the
268 OF model, we reduced litter size by cross-fostering to alter nutritional status, as described previously [23].
269 Because the stomach milk contents of the OF pups were approximately 2-fold higher than those of the
270 HFD and C pups, the calorie intake in the OF pups would be approximately 2.4-fold higher than that of

271 the HFD and C pups considering the energy content in milk. Consistent with previous studies, the
272 absolute intake of breast milk was elevated and somatic growth (body length and body weight) was
273 accelerated in overfed pups by reducing litter size [14].The present study showed no effect of maternal
274 high fat diet on the energy contents of the dam's milk. Nasser et al. demonstrated that maternal dietary fat
275 intake effects on medium and long chain fatty acids in human breast milk, which is consistent with our
276 results [32]. These results suggest that the alteration of dietary fat content could change breast milk fatty
277 acid composition even in a short period of time. Moreover, taken together with the results of the acylated
278 bioactive form of ghrelin in the stomach of the HFD pups, we can assume that the changes in breast milk
279 fatty acid composition might facilitate the growth of pups through the acylation of ghrelin (see below).
280 Neonatal offspring exposed to a maternal high-fat diet consumed more milk on PND 3 and 7, but the
281 differences disappeared by PND 10 without changing the body weight of the dams [37]. These results
282 suggested that the increase in caloric intake in HFD dams during lactation might be used for a purpose
283 other than maintenance of body weight. Neonatal food consists exclusively of breast milk until PND 14,
284 and has a dominant influence on the nutritional environment during the early postnatal period. The
285 fatty-acid composition of milk was altered when dams consumed a high-fat diet: in particular, the percent
286 composition of long-chain fatty acids was elevated, whereas the percent composition of medium-chain
287 fatty acid was reduced. The increased percent composition of oleic acid and linoleic acid may reflect the
288 high composition of the same fatty acid in the high-fat diet. The changes in fatty-acid composition in milk
289 might have directly influenced the concentration of these compounds in pup serum. A Recent human

290 study suggests that breast milk fatty-acid profile related to infant growth and body composition [31]. Both
291 maternal high-fat diet and overfeeding during the postnatal period resulted in a significant increase of
292 bodyweight in offspring, about 20 percent greater than pups in the Control group. Pups in reduced litters
293 exhibited elevated milk intake and body weight [7]. These results suggested that the effect of body weight
294 change between pups in HFD and C groups might be affected by milk nutrients, but not the volume of
295 milk intake.

296 In the fed status, blood glucose levels were elevated in the HFD and OF pups on PND 14, but only the
297 OF pups had hyperinsulinemia on PND 14 and 21. HOMA-IR levels were significantly higher in the HFD
298 and OF pups than in the C pups on PND 14, which indicates that the HFD and OF pups have impaired
299 insulin sensitivity compared to the C pups. Recently reports demonstrated that postnatal overfeeding
300 induced insulin resistance even in the postnatal period [4, 23]. HOMA-beta levels were significantly
301 higher in the OF pups than in the C pups. Based on the increased serum insulin levels and the
302 transcriptional up-regulation of insulin and Pdx-1 mRNA levels, it appears that the OF pups compensated
303 for the impairment in insulin sensitivity. The HFD pups tended to be impaired insulin sensitivity on PND
304 21. The insulin contents in the HFD pups increased compared with the C pups on PND 21, but there were
305 no significant differences in serum insulin levels between the HFD and C pups. Taken together, these
306 results suggested that the HFD pups had impaired beta-cell function and secretion. The HFD pups fed
307 milk with high n-6/n-3 polyunsaturated fatty acids (PUFA) ratio and had significantly higher serum n-6
308 PUFA (Linoleic acid and arachidonic acid) levels compared to the C pups. Wei *et al.* demonstrated that

309 transgenic expression of *mfat-1* that decrease in the n-6/n-3 tissue PUFA ratio in the islets enhanced
310 insulin secretion [42]. Dobbins *et al.* reported that rats fed high-fat diet enriched in linoleic acid
311 suppressed insulin output compared to rats fed enriched saturated fatty acid [11]. Therefore, there is a
312 possibility that the changes in the n-6/n-3 PUFA ratio accompanied by the increase in linoleic acid
313 consumption may cause alterations in beta-cell function and transcriptional changes in the pancreas of the
314 HFD pups.

315 Gut incretins, which play an important role in promoting insulin secretion, are regulated by nutrient
316 ingestion [2, 5, 12]. GLP-1, a posttranslational processing product of proglucagon in intestine, is encoded
317 by the proglucagon gene. Gene expression of proglucagon and GIP were increased in both OF and HFD
318 pups on PND 14. The content of GIP peptide increased significantly, and GLP-1 content had a tendency
319 to increase, in the jejunum of OF pups. In HFD pups, however, the levels of these peptides did not change
320 relative to those in C pups. Recently Hayashi et al [18] reported that GLP-1 production in the GLUTag
321 cell line is impaired by free fatty acids via endoplasmic reticulum stress and a reduction in the protein
322 levels of prohormone convertase 1/3, suggesting that the discrepancy between mRNA expression and
323 production of GLP-1 might happen in HFD pups. The differences in production of incretins between the
324 OF and HFD pups may relate to the differences in insulin secretion with insulin resistance. However,
325 further experiments would be required to explain the difference in production of both peptides and to
326 clarify the influence of these peptides on process glucose-induced insulin secretion in these postnatal
327 excessive weight models.

328 Circulated ghrelin levels decrease in adults [22] and are negatively correlated with BMI and insulin
329 resistance in childhood [22, 40]. In the HFD and OF pups, the acylated bioactive form of ghrelin in the
330 stomach was apparently elevated despite increased body weight and blood glucose elevation on PND 14.
331 This result suggested that dietary nutrition might have a primary effect on acyl modification of ghrelin
332 during the early postnatal period. Ghrelin can induce body weight gain [41] and modulate energy balance
333 [19]. Elevated ghrelin production in the early postnatal period may explain the weight gain in the HFD
334 and OF pups. Further study will be required to explain the relationship between milk nutrients and ghrelin
335 production in the early postnatal period.

5. Conclusions

In this study, two models of early postnatal excessive weight gain had different effects on glucose regulation and the production of pancreatic and gastrointestinal peptides. One model involved overfeeding pups, resulting in insulin resistance along with elevated blood glucose and serum insulin and an accompanying increase in incretin production. The other model involved pups of dams fed a high-fat diet (HFD). Maternal HFD during lactation modifies the fatty acid composition of breast milk, and pups in this group had elevated blood glucose accompanied by insufficient insulin and incretin production. Acylated ghrelin in the stomach was increased in both types of excessive weight gain pups. These findings suggest that the impact of obesity on glucose metabolism, including regulation of the insulin and insulinotropic polypeptides, differs significantly between neonatal and adult mice. Further work is required to clarify the role of glucose homeostasis in connecting early nutritional changes to obesity and specific diseases later in life.

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460 **Table 1. Primer sequences for RT-qPCR**

Gene	Primer		Accession number
36B4	sense	5'-TCATTGTGGGAGCAGACAATGTGGG-3'	NM_007475
	antisense	5'-AGGTCCTCCTTGGTGAACACAAAGC-3'	
Ins	sense	5'-TCAGCAAGCAGGTCATTGTTCC-3'	NM_008386
	antisense	5'-GAAGAAACCACGTTCCCCACAC-3'	
Gcg	sense	5'-CGTGCCCAAGATTTTGTGCAGTGGTTG-3'	NM_008100
	antisense	5'-TCTCGCCTTCCTCGGCCTTTCACCAGC-3'	
Gip	sense	5'-GAGGGGACTTTCATCAGTGATTACAG-3'	NM_008119
	antisense	5'-CAGGCCAGTAGCTCTTGAATCAGAA-3'	
Pdx-1	sense	5'-TACGCGGCCACACAGCTCTACAAG-3'	NM_008814
	antisense	5'-GGGCACTTCGTATGGGGAGATGTC-3'	
Ctrb	sense	5'-AATGACATCACCCTGCTGAAGCTGGCC-3'	NM_025583
	antisense	5'-GCCTGCTGCAGCTTGTCAGGGGTCTTG-3'	
Ghrl	sense	5'-ACCAGAAAGCCCAGCAGAGAAAGG-3'	NM_021488
	antisense	5'-ACTGAGCTCCTGACAGCTTGATGC-3'	
Goat	sense	5'-CACTGGATCCTGGACGACTC-3'	NM_001126314
	antisense	5'-GAGCTGTGCTTCGGTTCCACTGCCT-3'	

461

462

463 **Table 2. Fatty-acid composition of experimental diets (mg/100 kcal)**

	Fatty acid	Control diet	High-fat diet
C10:0	Caprylic acid	0.80	2.70
C12:0	Lauric acid	1.71	4.97
C14:0	Myristic acid	9.50	50.83
C16:0	Palmitic acid	172.40	950.08
C16:1	Palmitoleic acid	7.52	66.69
C18:0	Stearic acid	81.17	473.79
C18:1	Oleic acid	316.56	1802.79
C18:2	Linoleic acid	449.07	1380.93
C18:3	α-Linolenic acid	41.84	84.57
C20:4	Arachidonic acid	1.36	10.63
C20:5	Eicosapentaenoic acid	0.00	0.00
C22:6	Docosahexaenoic acid	0.00	0.00

464

465

466 **Table 3. Summary of nutrition content in milk**

Content	Control	HFD	OF	
Protein (g/100ml)	19.0 ± 5.7	18.3 ± 1.9	12.8 ± 0.7	
Fat (g/100ml)	14.58 ± 8.41	17.02 ± 8.04	19.55 ± 0.32	
Lactose (g/100ml)	2.98 ± 0.08	3.10 ± 0.6	1.06 ± 0.6	[b]
Energy (kcal/gram)	188.0 ± 48.9	190.8 ± 43.8	231.3 ± 3.0	

467 [b]$P < 0.01$, HFD or OF vs. Control,

468

469 **Table 4. Fatty acid composition in milk (% of total fatty acids)**

	Fatty Acid Composition	Control	HFD	OF
C8:0	Caprylic acid	0.22 ± 0.02	0.18 ± 0.01	0.17 ± 0.01
C10:0	Capric acid	6.30 ± 0.43	4.12 ± 0.22 [b]	5.13 ± 0.28
C12:0	Lauric acid	14.39 ± 0.81	7.34 ± 0.27 [c]	12.17 ± 0.45
C14:0	Myristic acid	19.53 ± 0.93	8.63 ± 0.24 [c]	16.65 ± 0.88
C16:0	Palmitic acid	27.66 ± 0.62	21.35 ± 0.56 [c]	27.72 ± 0.92
C16:1	Palmitoleic acid	1.70 ± 0.08	1.07 ± 0.02 [c]	1.29 ± 0.06 [b]
C18:0	Stearic acid	2.29 ± 0.15	4.21 ± 0.15 [c]	3.41 ± 0.03 [b]
C18:1	Oleic acid	16.32 ± 1.58	27.69 ± 0.57 [c]	20.79 ± 1.48
C18:2	Linoleic acid	7.42 ± 0.61	19.31 ± 0.23 [c]	8.07 ± 0.26
C18:3	α-Linolenic acid	0.41 ± 0.04	0.80 ± 0.01 [c]	0.44 ± 0.02
C20:2	Eicosadienoic acid	0.68 ± 0.10	1.34 ± 0.05 [c]	1.00 ± 0.15
C20:3	Dihomo-gamma-linolenic acid	0.37 ± 0.03	0.54 ± 0.03 [b]	0.80 ± 0.08
C20:4	Arachidonic acid	0.42 ± 0.06	0.70 ± 0.05	0.51 ± 0.02
C20:5	Eicosapentaenoic acid	0.06 ± 0.01	0.07 ± 0.00	0.56 ± 0.00
C22:6	Docosahexaenoic acid	0.15 ± 0.01	0.18 ± 0.02	0.06 ± 0.04 [a]
n-6		8.89 ± 0.77	22.19 ± 0.31 [c]	9.93 ± 0.30
n-3		0.61 ± 0.04	1.04 ± 0.03 [c]	0.74 ± 0.02
n-6/n-3		14.50 ± 0.38	21.24 ± 0.50 [c]	13.36 ± 0.12

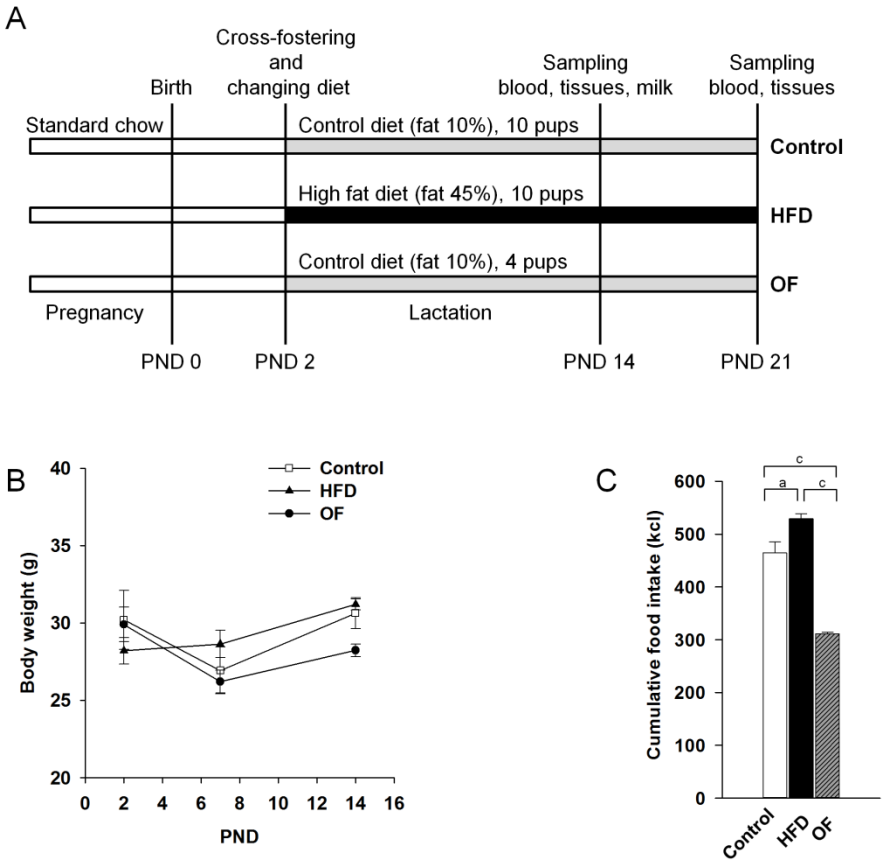
470 [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$ vs. Control dams.

471

472

473

474 **Figure 1**



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476

477 **Fig. 1.** Schematic of dietary intervention for the dams in three groups. Experimental procedures are

478 indicated according to the age of the pups (A). Changes in body weight and cumulative food intake of

479 dams in the Control, HFD, and OF groups during lactation. Body weight changes of the three groups on

480 PND 2, 7, and 14 (A). Cumulative food intake (kcal) calculated from PND 2 to 14 (B). Data are

481 means ± SE (Control, n = 3; HFD, n = 3; OF, n = 4). [a] $P < 0.05$, [c] $P < 0.001$.

482

Figure 2

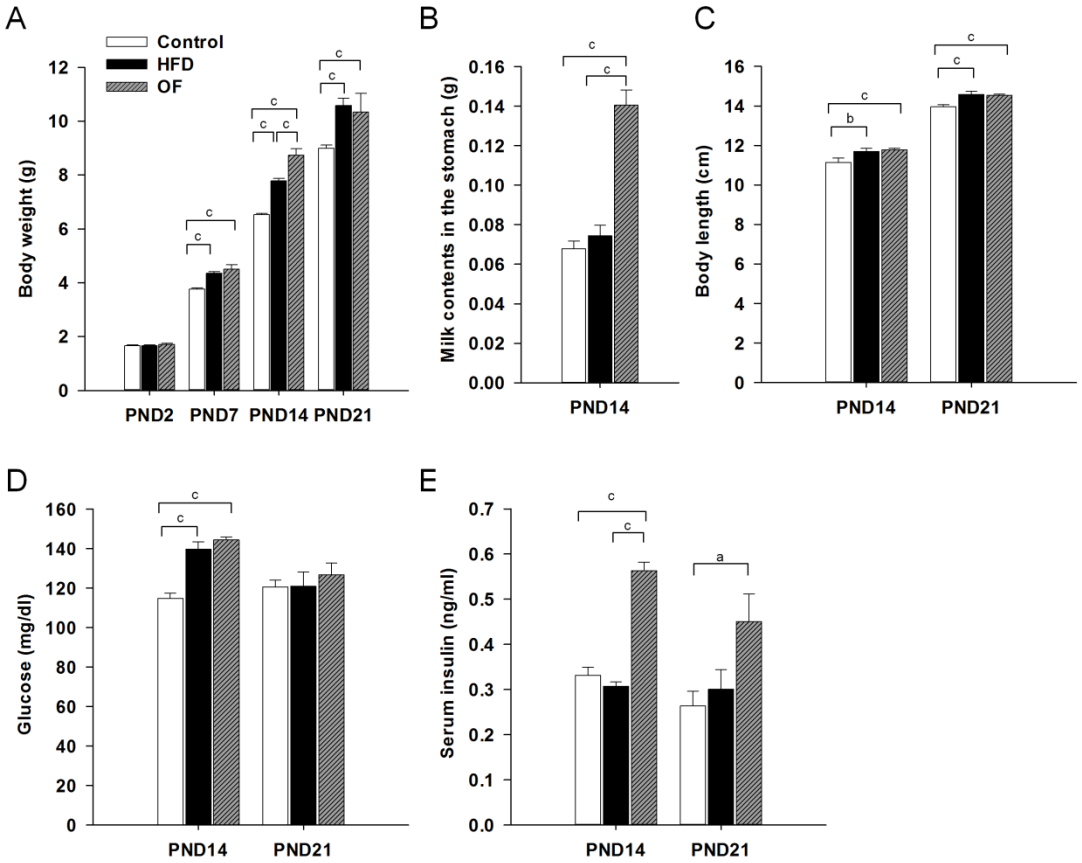


Fig. 2. Body weight (A), milk contents in the stomach (B), body length from the nose to the base of the tail (C), blood glucose levels (D), and serum insulin levels (E) of pups of dams in the C, HFD, and OF groups during lactation. Data are means ± SE (Control, n = 30; HFD, n = 30; OF, n = 16 on PND 7 and 14: Control, n = 10; HFD, n = 10; OF, n = 8 on PND 21 in Figure A, C, and D. Control, n = 40; HFD, n = 39; OF, n = 18 on PND 14 in Figure B.). [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Figure 3

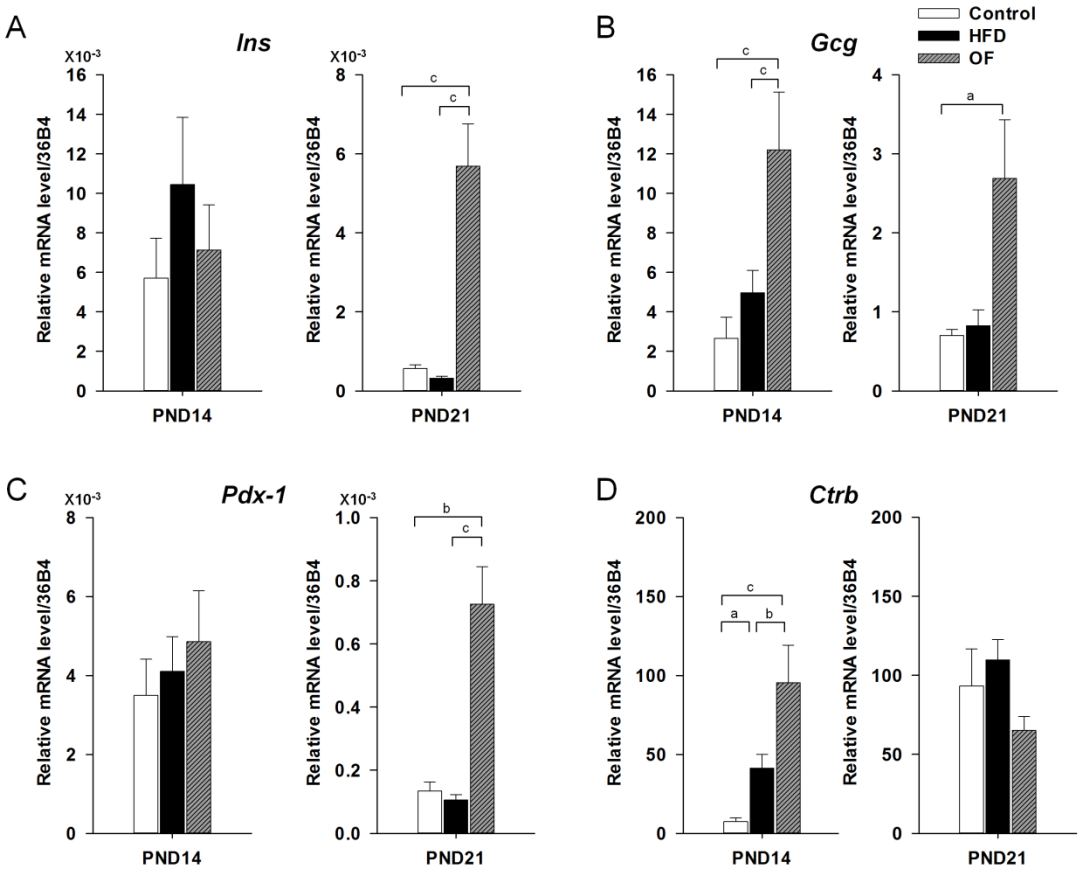


Fig. 3. Gene-expression analysis of insulin (*Ins*) (A), proglucagon (*Gcg*) (B), pancreatic and duodenal homeobox 1 (*Pdx-1*) (C) and chymotrypsinogen B1 (*Ctrb*) (D) in the pancreas of pups from dams in the Control, HFD, and OF groups during lactation. Data are means ± SE (Control, n = 17–19; HFD, n = 20; OF, n = 8 on PND 14: Control, HFD and OF, n = 4 on PND 21). [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Figure 4

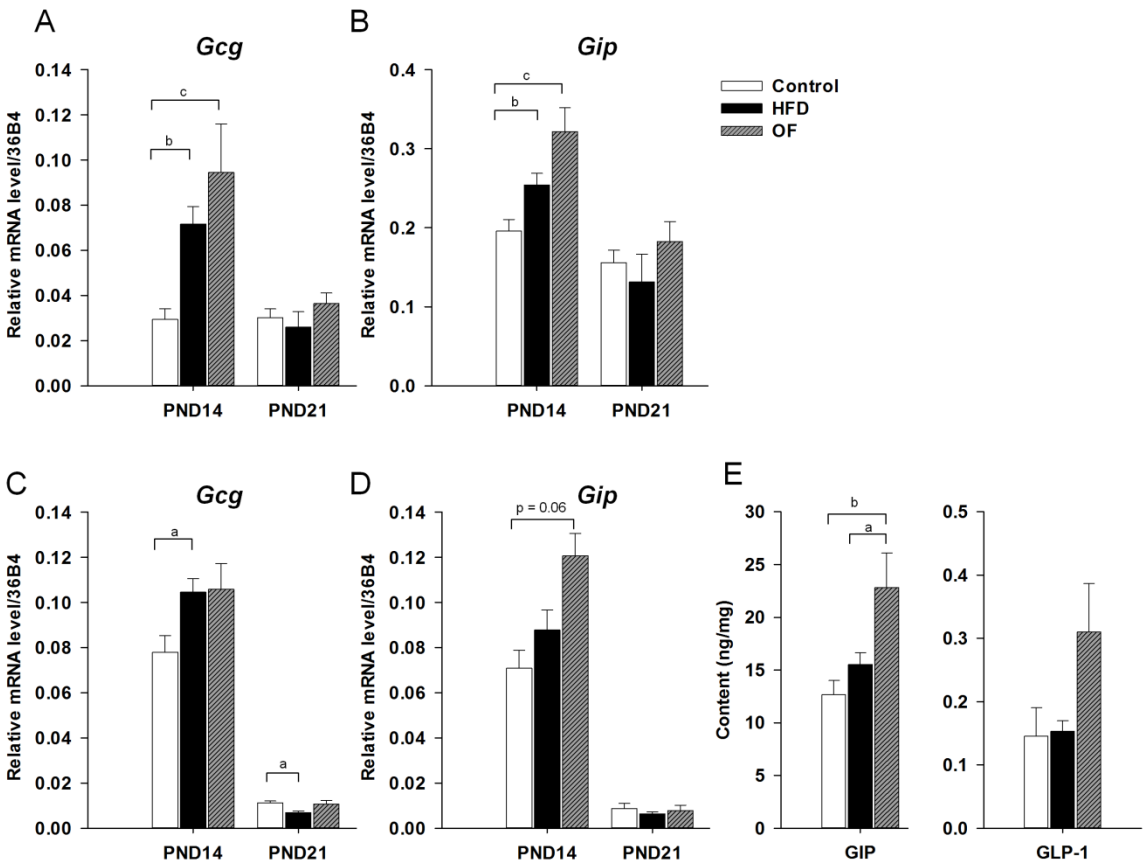


Fig. 4. Gene-expression analysis of glucagon/GLP-1 (*Gcg*) (A, C) and GIP (*Gip*) (B, D) in the jejunum (A, B) and ileum (C, D) of pups from dams in the Control, HFD, and OF groups during lactation (Control, n = 20; HFD, n = 19–20; OF, n = 8 on PND 14: Control, n = 10; HFD, n = 10; OF, n = 8 on PND 21). The contents of GIP and GLP-1 in the jejunum on PND 14 (Control, n = 17; HFD, n = 16; OF, n = 7) (E). Data are means ± SE. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Figure 5

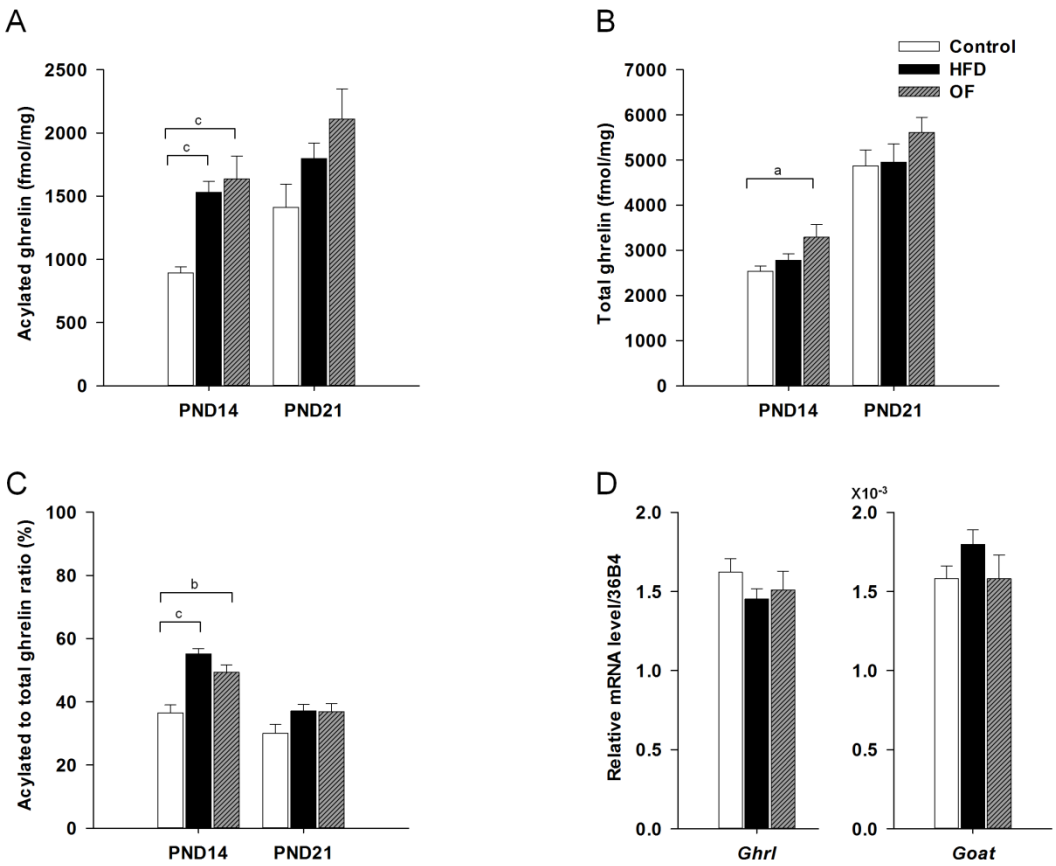


Fig. 5. Stomach ghrelin synthesis in pups from dams in Control, HFD, and OF groups during lactation. The contents of acylated ghrelin (A) and total ghrelin (B) and the ratio of acylated ghrelin to total ghrelin (C) were measured using two types of specific RIA. Gene-expression analysis of ghrelin (*Ghrl*) and GOAT (*Goat*) (D). Data are means ± SE (Control, n = 20; HFD, n = 19–20; OF, n = 8 on PND 14: Control, n = 10; HFD, n = 10; OF, n = 8 on PND 21). [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Figure 6

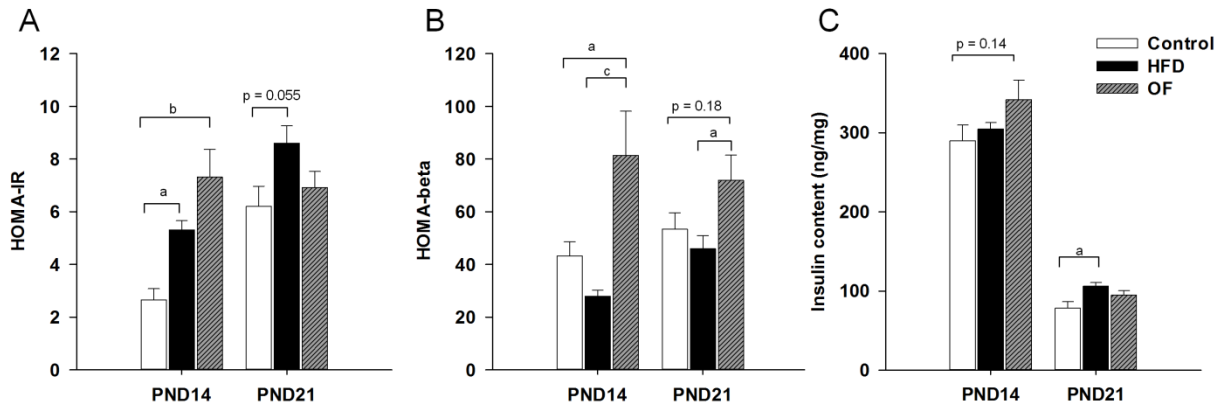
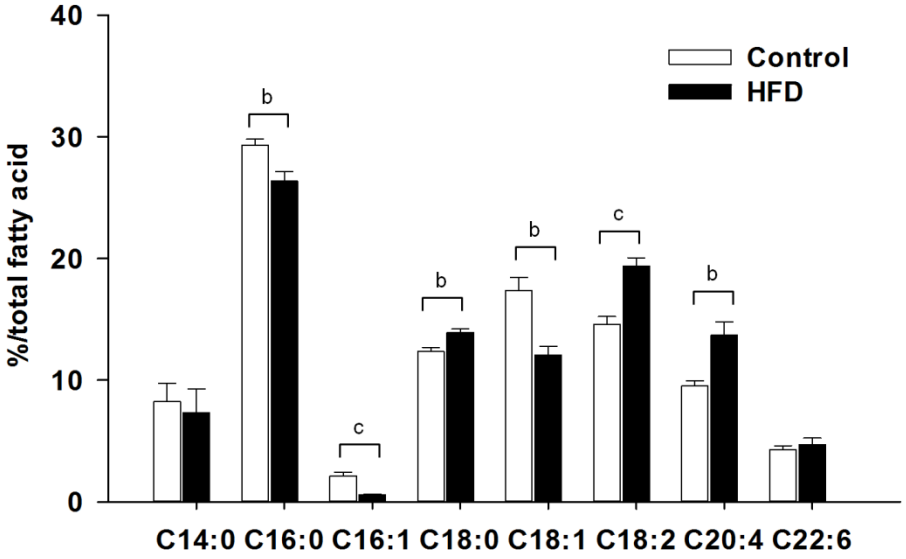


Fig. 6. Assessment of insulin sensitivity (HOMA-IR, A) and beta-cell function (HOMA-beta, B), and the insulin contents of pancreas in 4-h fasted pups of dams in the C, HFD, and OF groups during lactation. Data are means ± SE (Control, n = 10; HFD, n = 10; OF, n = 8 on PND 14: Control, n = 9; HFD, n = 10; OF, n = 8 on PND 21). [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

526 **Figure 7**



527

528 **Fig. 7.** Serum fatty acid compositions in Control and HFD pups on PND 14. Results were expressed as

529 percentages of total fatty acids. Data are means ± SE (Control and HFD, n = 6). [b] $P < 0.01$, [c] $P < 0.001$.

530