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# Association between prepartum metabolic status and resumption of postpartum ovulation in dairy cows

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ABSTRACT

Cows transitioning from late gestation to early lactation experience an increase in energy demands, which lead to a negative energy balance (NEB) because the greater energy requirement is not fully synchronized with the intake of dry matter. In this context, there is an increase in plasma NEFA and ghrelin concentrations and a decrease in plasma insulin and glucose-dependent insulinotropic polypeptide (GIP) concentrations. This situation could have a negative impact on the return to cyclicity because some of these variables have been associated with reduced GnRH and LH pulsatility (high NEFA and low insulin concentrations). However, there are no studies showing the relationship between ghrelin or GIP and reproductive performance. It is known that these hormones are related with lipolysis and NEB, with NEB being one of the main determinants of GnRH pulse generator activity. Thus, the objective of the present study was to evaluate the association between plasma NEFA concentration and metabolic hormones (insulin, ghrelin, and GIP) before parturition and their associations with the resumption of postpartum ovulations in dairy cows. A completely randomized block design was used in a commercial dairy herd with sampling day (visit to farm) as the blocking criteria. Holstein cows (n = 92) were screened for plasma NEFA concentration −5 d (±2 d) relative to the expected parturition day, and top and bottom quartiles were considered as high (H-NEFA) and low (L-NEFA) NEFA groups. Data were analyzed with correlation, linear regression, and proportional hazard regression models. Plasma NEFA concentration (H-NEFA mean = 294 μM, SD = 141.2; and L-NEFA mean = 122 μM, SD = 25.3) was correlated ($P < 0.01$) with plasma insulin (r = −0.374) and ghrelin (r = −0.346) concentrations but not with plasma GIP concentration ($P = 0.64$). The greater the concentration of insulin, the lesser the prepartum NEFA concentration (for each 1 μU/mL of plasma insulin increase, there is a decrease of 1.223 ± 0.62 μM of NEFA). Plasma ghrelin and GIP concentrations were not associated with plasma NEFA concentration. Finally, H-NEFA prepartum cows were less likely to resume ovulation than L-NEFA cows (hazard ratio [HR] = 0.563, 95% confidence interval [CI] = 0.314–1.011), whereas high ghrelin cows were more likely to resume ovulation than low ghrelin cows (HR = 1.873, 95% CI = 0.846–4.145). Conversely, resumption of ovulation was not associated with prepartum insulin and GIP concentrations. Prepartum NEFA and possibly ghrelin are associated with the return to postpartum cyclicity; however, insulin and GIP are not related to the resumption of ovulation in dairy cows.



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## 1. Introduction

Transition from nonlactating pregnant state to nonpregnant lactating state is characterized by a sharp rise in energy demand to sustain milk production that is not fully synchronized with a concomitant increase in dry matter intake [1,2]. This leads to a negative energy balance (NEB) that is characterized by high NEFA [3], low insulin [4], low glucose-dependent insulinotropic polypeptide (GIP; [5]), and high ghrelin plasma concentrations [6]. The relationship between NEFA concentration and these metabolic hormones has only been reported in postpartum dairy cows [5,6], but there is no information about the association of NEFA and these hormones before parturition. High plasma NEFA concentration has also been associated with a delayed resumption of postpartum ovulation in dairy cows [7,8] and also with poor reproductive performance [9–11]. Some studies evaluating the relationship between metabolic status and the onset of ovarian cyclicity have measured insulin [8,12,13], but none of them have included ghrelin and GIP. This aspect would be interesting because ghrelin is related to NEB [6,14] and GIP is associated with lipolysis [15]. In fact, there is a consensus that NEB is one of the main determiners of GnRH pulse generator activity [12,16], given that low plasma insulin concentration [16,17] and high NEFA lead to reduced GnRH and LH pulsatility [18–20]. In addition, high plasma NEFA concentration (and GH) would cause an insulin resistant state associated with reduced sensitivity to LH and FSH in the ovaries [21]. Finally, Leroy et al [22] reported that high NEFA concentration could also have a direct toxic effect on oocytes and suggested that this toxicity could further compromise fertility in cows.

As mentioned previously, there are no studies evaluating the association between precalving concentrations of these metabolic hormones with plasma NEFA concentration and their impact on subsequent fertility in dairy cows. The main hypothesis to test was that plasma NEFA concentration is correlated with plasma insulin, GIP, and ghrelin concentrations before parturition in dairy cows. We also hypothesized that high insulin, high GIP, and low ghrelin, accompanied by low NEFA, are associated with a shorter interval for return to postpartum cyclicity in lactating dairy cows. Therefore, our first objective was to assess the association between NEFA and metabolic hormones (ie, insulin, ghrelin, and GIP) before parturition in dairy cows, and our secondary objective was to evaluate the association among all these metabolic indicators (NEFA, insulin, ghrelin, and GIP) before calving with the resumption of postpartum ovulations in dairy cows.

## 2. Materials and methods

### 2.1. Animals, feeding, and management

The experimental procedures were approved by the Agricultural Animal Care and Use Committee of The Ohio State University (IACUC # 2016A00000069). The study was conducted from August to November 2016 in 92 prepartum multiparous Holstein cows in a commercial dairy herd (Marshallville, OH). During the dry period, cows were fed with a mixed diet constituted by 69% forage and 31% concentrate (Table 1). After parturition, cows received a lactation diet, which was a mixture of haylage (53.67% dry matter [DM], 18.77% CP, and 1.433 Mcal NEl/kg), commercial premix concentrate (76.10% DM, 22.97% CP, and 0.7879 Mcal NEl/kg), and corn silage (35% DM, 6.81% CP, and 1.5653 Mcal NEl/kg). During the entire study, cows were housed in a free stall barn with permanent access to fresh water. None of the animals in this experiment suffered any postpartum clinical disorder.

Pregnant nonlactating cows (n = 92) were screened for plasma NEFA concentration on d −5 (±2 d) based on predicted parturition day in 3 visits (blocks) to the farm (August 11, August 25, and September 15, respectively). All 92 cows were used to evaluate the prepartum association between plasma NEFA and metabolic hormone concentrations prepartum (ghrelin, GIP, and insulin). In addition, at each visit, 9 cows in the highest quartile and 9 cows in the lowest quartile of plasma concentration of NEFA (n = 54 cows total) were considered as high (mean = 294 μM, SD = 141.2; H-NEFA; n = 27) and low (mean = 122 μM, SD = 25.33; L-NEFA; n = 27) NEFA groups and used to assess the relationship between prepartum metabolic status and resumption of postpartum ovulations.

### 2.2. Sampling

Individual feed ingredient samples were collected twice before and once after parturition, and they were subsequently analyzed for chemical composition at the Rock River Laboratory Inc, Watertown, WI. Blood samples were taken 30 min before the morning feeding from the tail vessels of prepartum cows (−21 to −1 d relative to calving date) to measure plasma concentrations of NEFA, insulin, ghrelin, and GIP. Blood was collected in 12 mL tubes containing 200 mL EDTA and kept on ice bath during the sampling. After centrifugation for 25 min (1,800× *g* at 4°C), plasma was aliquoted into individual polypropylene tubes and stored at −80°C until analysis. Milk was sampled twice a week from 15 through 56 d in milk during the midday milking. Milk was mixed, and then an aliquot was placed in plastic tubes containing preservative for milk composition, and another one was put in polystyrene tubes and kept on ice bath during the sampling. Once in the laboratory, samples were pipetted into microcentrifuge tubes and stored at −20°C until analysis for progesterone. Milk yield, milk composition, and milk progesterone concentration were recorded for each cow at the midday milking.

**Table 1**
Diet formulation and composition.

| Ingredient | DM (kg) | CP (%) | NEl (Mcal/kg) |
|---|---|---|---|
| Grass hay | 0.79 | 6.78 | 1.4043 |
| Ground straw | 0.83 | 3.37 | 0.9744 |
| Soybean meal | 1.96 | 56.53 | 2.0834 |
| Prefresh supplement | 1.55 | 16.25 | 1.2169 |
| Corn silage | 6.19 | 6.83 | 1.4925 |

Abbreviation: DM, dry matter.

### 2.3. Laboratory analysis

Plasma NEFA, glucose and milk progesterone concentrations were measured within a week of sampling, and plasma insulin, GIP, and ghrelin were measured within 1 yr of the sample collection.

Blood plasma samples were analyzed for NEFA and glucose with a commercial kit (Wako Diagnostics, Mountain View, CA and Stanbio Laboratory, Boerne, TX, respectively). Intra-assay CV accepted was <10% for each metabolite. Plasma insulin concentration was measured using RIA (Porcine RIA #PI-12K; EMD Millipore Corporation, Billerica, MA). A validation for the porcine assay and bovine plasma was conducted based on parallel displacement of insulin binding by incremental addition of bovine plasma and compared with an insulin standard curve and by recovery of swine insulin on bovine plasma (97 ± 5% recovery). Sensitivity of the insulin assay (the concentration at which bound counts were 90% of binding for the zero standard) was 3.125 μU/mL. The intra-assay CV was 6.8%. Plasma GIP concentration was measured using RIA, as described by Relling and Reynolds [5]. The GIP assay used was based on the disequilibrium assay described by Morgan et al [23]. The assay used the insulin assay buffer and 24- and 48-h incubations before additions of labeled GIP and a second antibody, respectively. Using the primary antibody described by Larsen et al [24], displacement of labeled human GIP (T-027–02; Phoenix Pharmaceuticals, Inc) binding by serial additions of bovine plasma was parallel to the displacement by serial additions of porcine GIP standards (Sigma-Aldrich, St. Louis, MO). The intra-assay CV was 9.8%, and the minimum sensitivity (90% of zero standard binding) was 0.003 pmol per tube (0.015 pmol/mL). Plasma ghrelin concentration was measured using an octanoylated ghrelin kit (Active Ghrelin Kit GHRA-88HK; LINCO Research, St. Charles, MO), as described in a study by Relling et al [25]. Briefly, immediately after thawing, the plasma samples (500 μL) used for ghrelin analysis were acidified with 25 μL of 1 M HCl and 5 μL of phenylmethylsulfonyl fluoride (10 mg/mL), as recommended in the kit protocol to decrease the breakdown of active ghrelin. Sample analysis was performed as described by Bradford et al [26]. Milk progesterone concentration was measured using a commercial nonextraction solid-phase bovine progesterone microplate kit (BioMetallics, Princeton, NJ) following the manufacturer's instructions. A cow was considered as cycling when milk progesterone concentration was above 3 ng/mL for 2 consecutive samplings [27].

### 2.4. Experimental design and statistical analysis

The study was run with a completely randomized block design where visit to farm (n = 3) and sampling day prepartum (n = 17) served as the blocking criteria. Sample size for plasma NEFA concentration (n = 54) was estimated as the number of cows needed to detect a difference of 10 d (with a pooled SD of 15 d) between means in the interval from calving to resumption of postpartum ovulation, with 80% of power and 1-sided 95% of confidence assuming equal group size [28].

Partial Pearson correlations (n = 92) among prepartum plasma NEFA, insulin, ghrelin, and GIP concentrations were estimated with Proc Corr of SAS (SAS/STAT ver. 9.4; SAS Institute Inc, Cary, NC) using sampling day as the partial option. The effects of plasma insulin, ghrelin, and GIP concentrations on prepartum plasma NEFA concentration were evaluated with Proc Glimmix of SAS with normal distribution and identity link function, restricted maximum likelihood estimation technique, and Kenward–Roger method. Models included the fixed effect of insulin, ghrelin, and GIP concentrations and their interaction as continuous predictors, and the random effects of block (visit) and sampling day (day prepartum). Univariable models were run first, and predictors having $P < 0.2$ were offered to the multivariable model where they remained if $P < 0.15$. Statistical significance was set at $P < 0.05$, and a tendency for significance was set at $P < 0.10$.

The hazard of resumption of postpartum ovulation was estimated with Proc PHREG of SAS. Models included the fixed effect of prepartum NEFA (low: <175 μM vs high: ≥175 μM; n = 27 per group), insulin (low: <15.25 μU/mL vs high: ≥15.25 μU/mL; n = 27 per group), ghrelin (low: <166 pg/mL vs high: ≥166 pg/mL; n = 27 per group), and GIP (low: <48.80 μM vs high: ≥ 48.80 μM; n = 27 per group) and the random effect of block (visit). The groups were divided by the median concentrations of NEFA or each hormone. Univariable models were run first, and predictors having $P < 0.2$ were then offered to the multivariable model. Multivariable modeling was performed using a manual backward elimination method with an exclusion criterion set at $P > 0.15$. Median (95% confidence interval [CI]) days from calving to resumption of postpartum ovulation were estimated with Proc Lifetest of SAS.

## 3. Results

### 3.1. Metabolic indicators

Nonesterified fatty acid was negatively correlated with insulin (r = −0.374, $P < 0.01$; Table 2) and ghrelin (r = −0.346, $P < 0.01$; Table 2) but not with GIP (r = −0.055, $P = 0.64$), whereas plasma insulin and ghrelin concentrations were positively associated (r = 0.350, $P < 0.01$; Table 2). Finally, plasma insulin and ghrelin concentrations were not correlated with GIP ($P > 0.1$). Univariable and multivariable linear regression models showed that the greater the concentration of insulin, the lesser prepartum NEFA concentration (for each 1 μU/mL of increase in plasma insulin concentration, there is a decrease of 1.486 ± 0.62 μM of plasma NEFA concentration; $P = 0.02$, Table 3). The remaining predictors did not explain NEFA concentration ($P > 0.1$).

**Table 2**
Partial correlation[a] coefficients (*P*-values) among concentration of NEFA, insulin, ghrelin, and GIP in prepartum dairy cows (n = 92).

| | Insulin | Ghrelin | GIP |
|---|---|---|---|
| NEFA | −0.374 ($P < 0.01$) | −0.346 ($P < 0.01$) | −0.055 ($P = 0.635$) |
| Insulin | | 0.350 ($P < 0.01$) | −0.047 ($P = 0.686$) |
| Ghrelin | | | −0.176 ($P = 0.129$) |

Abbreviation: GIP, glucose-dependent insulinotropic polypeptide.
[a] Sampling day prepartum (−21 through −1) was used as partial in Proc Corr of SAS.

**Table 3**
Effect of insulin, ghrelin, and GIP on plasma NEFA concentration in prepartum dairy cows (n = 92).[a]

| | Estimate[b] | SE | *P*-values |
|---|---|---|---|
| Intercept | 229.41 | 38.56 | 0.01 |
| Insulin | −1.486 | 0.62 | 0.02 |
| Ghrelin | −0.158 | 0.19 | 0.40 |
| GIP | −0.276 | 0.94 | 0.77 |

Abbreviation: GIP, glucose-dependent insulinotropic polypeptide.
[a] Fixed effects were estimated with Proc Glimmix of SAS with normal distribution and identity link function with random effects of block (farm visit) and sampling day prepartum. Univariable models were run first, and then predictors having $P < 0.2$ were offered to multivariable linear model where they remained if $P < 0.15$.
[b] NEFA concentration was expressed in μM/L. Changes in NEFA are expressed per unit of change in predictor concentration (μU/mL of insulin, pg/mL of ghrelin, and μM of GIP).

### 3.2. Resumption of postpartum ovulation

Resumption of postpartum ovulation was associated with prepartum NEFA ($P = 0.05$) and ghrelin ($P = 0.09$), but not with insulin ($P = 0.34$) and GIP ($P = 0.20$; Table 4). According to multivariable analysis, cows having greater plasma NEFA concentration had a lower hazard of resumption than cows with low NEFA concentration (hazard ratio [HR] = 0.563, $P = 0.06$), whereas cows with high concentration of ghrelin had a tendency for a higher hazard of ovulation than cows with low ghrelin concentration (HR = 1.873, $P = 0.10$; Table 5). Finally, plasma insulin and GIP concentrations were not associated with the hazard of ovulation ($P > 0.15$; Table 5).

**Table 4**
Association of prepartum concentration of NEFA, insulin, ghrelin, and GIP with the resumption of postpartum ovulation in dairy cows (n = 54) assessed with univariable proportional hazard regression models.

| | Resumption of postpartum ovulation | | | |
|---|---|---|---|---|
| | Days[a] | HR[b] | 95% CI | *P* |
| NEFA[c] | | | | 0.047 |
| Low | 33 (24–42) | 1 | | |
| High | 44 (27–47) | 0.555 | 0.311–0.993 | |
| Insulin[d] | | | | 0.344 |
| Low | 41 (26–47) | 1 | | |
| High | 32 (24–46) | 1.324 | 0.741–2.366 | |
| Ghrelin[e] | | | | 0.088 |
| Low | 40 (27–47) | 1 | | |
| High | 32 (24–46) | 1.975 | 0.904–4.317 | |
| GIP[f] | | | | 0.198 |
| Low | 36.5 (26–43) | 1 | | |
| High | 39 (26–47) | 0.670 | 0.357–1.257 | |

Abbreviations: CI, confidence interval; GIP, glucose-dependent insulinotropic polypeptide; HR, hazard ratio.
[a] Days: median (95% CI) days from calving to resumption of postpartum ovulation estimated with Proc Lifetest of SAS.
[b] HR estimated with Proc PHReg of SAS (univariable models).
[c] NEFA: prepartum NEFA concentration dichotomized by the median value (175 μM).
[d] Insulin: prepartum insulin concentration dichotomized by the median value (15.25 μU/mL).
[e] Ghrelin: prepartum ghrelin concentration dichotomized by the median value (166 pg/mL).
[f] GIP: prepartum GIP concentration dichotomized by the median value (60.52 μM).

**Table 5**
Association of prepartum concentration of NEFA, insulin, ghrelin, and GIP with the resumption of postpartum ovulation in dairy cows (n = 54) assessed with a multivariable proportional hazard regression model.

| | Resumption of postpartum ovulation | | | |
|---|---|---|---|---|
| | Days[a] | HR[b] | 95% CI | *P* |
| NEFA[c] | | | | 0.054 |
| Low | 33 (24–42) | 1 | | |
| High | 44 (27–47) | 0.563 | 0.314–1.011 | |
| Ghrelin[d] | | | | 0.107 |
| Low | 40 (27–47) | 1 | | |
| High | 32 (24–46) | 1.873 | 0.846–4.145 | |

Abbreviations: CI, confidence interval; GIP, glucose-dependent insulinotropic polypeptide; HR, hazard ratio.
Prepartum insulin concentration dichotomized by the median value (15.25 μU/mL) and prepartum GIP concentration dichotomized by the median value (60.52 μM) were removed from multivariable model ($P > 0.15$) and Table 2.
[a] Days: Median (95% CI) days from calving to resumption of postpartum ovulation estimated with Proc Lifetest of SAS.
[b] HR estimated with Proc PHReg of SAS (multivariable model including the fixed effect of NEFA, insulin, ghrelin, and GIP and also the random effect of block).
[c] NEFA: prepartum NEFA concentration dichotomized by the median value (175 μM).
[d] Ghrelin: prepartum ghrelin concentration dichotomized by the median value (166 pg/mL).

## 4. Discussion

Our results partially support the main hypothesis stating that prepartum plasma NEFA concentration was correlated with plasma concentration of metabolic hormones (insulin and ghrelin). As expected, plasma concentrations of NEFA and insulin were negatively correlated. Insulin is known to decrease lipolysis and consequently to reduce plasma NEFA concentration [3]. Surprisingly, plasma NEFA and ghrelin concentrations were also negatively correlated. Owing to the results reported by Bradford and Allen [6] and Roche et al [14] in their publications, we expected that NEFA and ghrelin would be positively correlated because plasma ghrelin concentrations are greater during NEB according to those authors. However, the results obtained in the present trial showed that the relationship is completely different. Tschop et al [29] demonstrated an increase in adiposity when ghrelin was injected to male mice. This increase in adipogenesis was assumed to be due to an increase in lipogenesis in the adipose tissue [29]. We assumed that this increase in adipogenesis happened in the present study, but there are no other works showing a direct effect of ghrelin in the adipose tissue in cows. Conversely, plasma NEFA and GIP concentrations were not correlated. It is possible that the secretion and action of GIP might be dependent on a glucose threshold for the GIP-secreting cells or may be because that relationship is also dependent on insulin concentration [30]. Further research is needed to understand the exact mechanism by which these hormones are related.

The results of this experiment support our secondary hypothesis stating that prepartum metabolic indicators are associated with the resumption of postpartum ovulations in dairy cows, given that we observed that L-NEFA and high ghrelin cows had a higher hazard (daily risk) of returning to postpartum cyclicity than their herd mates. These results

agree with those presented by Giuliodori et al [7], who detected that the higher the NEFA, the greater the risk for delayed ovulation and the lower the hazard of resumption of ovulations in postpartum dairy cows. According to the proportional hazard model, a cow with L-NEFA prepartum is 1.8 times more likely to ovulate next compared with a cow with H-NEFA prepartum (1/0.555 = 1.8; [31]). We expected that insulin concentration explained the resumption of ovulations as shown by Butler et al [13], but possibly this effect is only observed in postpartum dairy cows. Although Schneider [19] and Wade and Jones [32] stated that the greater plasma concentration of ghrelin could inhibit LH secretion and copulatory behavior in rats, the present study showed the opposite result, given that the greater prepartum ghrelin concentration was associated with a shorter interval of return to postpartum cyclicity in dairy cows. In this study, the 95% CI of the HR for plasma NEFA concentration (0.314 to 1.011) strongly suggest a negative effect whereas the 95% CI for plasma ghrelin concentration (0.846–4.145) indicates a positive effect. Therefore, prepartum plasma NEFA and ghrelin concentrations are associated with the hazard of resumption of postpartum ovulation in dairy cows (Table 5).

In conclusion, plasma NEFA concentration was negatively correlated with plasma insulin and ghrelin concentrations. We suggest that ghrelin could have a lipogenic effect in prepartum dairy cows, similar in effect to insulin but with less strength. Also, as prepartum cows having low NEFA and high ghrelin concentrations had a higher hazard (daily risk) of returning to postpartum cyclicity than their herd-mates, the resumption of postpartum ovulation is associated with the metabolic status of dairy cows before calving.

## CRediT authorship contribution statement

**E. Miqueo:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **A. Chiarle:** Methodology. **M.J. Giuliodori:** Methodology, Writing - original draft. **A.E. Relling:** Project administration, Methodology, Writing - original draft, Writing - review & editing.

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The authors declare no conflicts of interest.

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