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Do concentrations of non-esterified fatty acids change during gestation and lactation in healthy bitches?

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Abstract

During the gestation and lactation period, the energy demand in pregnant and lactating bitches is elevated. Non-esterified fatty acids (NEFAs) are utilized either directly from the fed diet or from body fat storage. High NEFA concentration in the blood plasma leads to an increased risk for diseases. Therefore, measuring blood NEFA concentrations may be an indicator for a period of scarcity. The aim of this study is to explore if serum NEFA concentrations in healthy bitches change during gestation and lactation. Healthy pregnant and lactating bitches were sampled on three appointed dates around parturition. NEFA values were examined with a multiparameter clinical chemistry analyser. All statistical analyses were performed using R. Overall, 38 bitches were enrolled in the study. Twenty-one bitches were sampled on all three appointed dates. The median NEFA concentration antepartum was 0.73 mmol/L (IQR: 0.59, 1.01); during peak lactation, it was 0.57 mmol/L (IQR: 0.44, 0.82); and around weaning, it was 0.58 mmol/L (IQR: 0.46, 0.73). NEFA concentrations rose slightly with litter size in late gestation. Body condition score had no influence on observed NEFA values. We conclude that NEFA concentrations widely remain within reference ranges in well-fed pregnant and lactating bitches. Nevertheless, they may be a valuable parameter to assess the actual metabolic status of malnourished pregnant and lactating bitches.

KEYWORDS

blood NEFA concentration, canine, energy demand, fat metabolism, free fatty acids, NEFA, reference interval (RI), scarcity indicator

1 | INTRODUCTION

An important factor for the health of the pregnant and lactating bitches and an appropriate development of her puppies is a sufficient supply with nutrients (Arlt et al., 2023; Johnson, 2008; Scantlebury et al., 2001; Wright-Rodgers et al., 2005). During the first weeks of canine gestation, the energy demand does not substantially increase. In the final trimester of gestation, however, it increases to the 1.25- to 1.5-fold demand compared to the basic need (Greco, 2008). During lactation, the energy demand increases even further, depending on the number of puppies, up to the 1.5- to 3-fold of the basic need (Greco, 2008; Mosier, 1977). However, in a concrete case, it may be difficult to assess the actual energy demand in the different stages of gestation and lactation as several factors such as bodyweight, breed, number of puppies, day of gestation or lactation are influencing factors. Therefore, it may be challenging for owners and breeders to provide the optimal type and amount of food in the different stages of gestation and lactation to their bitches.

Malnutrition can lead to impaired fetal development resulting in reduced birthweights and increased puppy mortality (Calabrò et al., 2021; Fontaine, 2012; Gianluppi et al., 2020; Kuhlman & Rompala, 1998; Ontko & Phillips, 1958; Scantlebury et al., 2001). It has recently been shown that several metabolites in the blood serum undergo substantial changes during gestation and lactation in bitches (Arlt et al., 2023). According to Calabrò et al., particular attention must be paid to the essential fatty acids profile (linoleic, α-linolenic, arachidonic acids) and vitamins because these nutrients affect ovarian hormone production, uterine protein production, placentation and fetal development (Calabrò et al., 2021). Furthermore, bitches fed semi-purified diets containing only small amounts of protein, delivered puppies with lower body weights at birth and a decreased survival rate (Ontko & Phillips, 1958). A correlation has been shown between feeding low carbohydrate diets and the number of puppies born alive and their survival rate 3 days after whelping (Romsos et al., 1981). Five bitches fed with a low carbohydrate diet whelped early which was not observed in the control group. In addition, free fatty acids in blood serum were increased by more than 50% during the last 2 weeks of gestation and remained high during lactation in the bitches fed the low carbohydrate diet (Romsos et al., 1981). In the clinical context, it has been stated that, in the first days after parturition, hypoglycaemia and dehydration are the major non-infectious causes for puppy mortality (Münnich & Küchenmeister, 2014). Therefore, it is essential for puppies' well-being and survival that the dam provides them with appropriate milk amount and composition.

In several species, measurement of non-esterified free fatty acids has been shown to be a good indicator of the actual energy supply (Brinkmann et al., 2013; Diez et al., 2004; Duffield et al., 2009; Kaneene et al., 1997; Minuti et al., 2014; Mustonen et al., 2009). In domestic European polecats (*Mustela putorius*), NEFA concentrations rose during a 5-day fast (Mustonen et al., 2009). The polecats studied exhibited an obese phenotype similar to that seen in overweight humans, domestic animals and seasonally obese wild mammals. These polecats were neither pregnant nor lactating. NEFA concentrations were measured only on the fifth day of fasting. Following this, the polecats were euthanized as part of the study. In dairy cows, negative energy balance due to increased energy demand, decreased food intake and suboptimal composition around calving and early lactation is a well-known phenomenon (Gerloff, 2000). Several authors reported on the advantage of measuring NEFA concentration to assess the risk of disease (LeBlanc et al., 2005; Ospina et al., 2010).

In a recent study, the NEFA reference interval (RI) for non-pregnant healthy female and male dogs has been determined (Doll et al., 2022). This research explores if serum NEFA concentrations in healthy bitches change during gestation and lactation, even if these are subjectively fed appropriate diets by the owners.

2 | MATERIALS AND METHODS

Healthy bitches during gestation and lactation were enrolled in the study. During routine visits for ovulation timing, owners of privately owned dogs visiting the Clinic for Animal Reproduction, Free University of Berlin, Germany, or Gemeinschaftspraxis Kreher, Stamnitz, Luckenwalde, Germany, were asked to participate in the study. Written informed consent was obtained from each owner. The project was reviewed and approved by the Landesamt für Gesundheit und Soziales Berlin, Germany (Reg 0165/16).

The included bitches were sampled on three appointed dates:

- 10 ± 2 days antepartum (expected date of birth based on date of ovulation +65 days)
- 21 ± 2 days post-partum (estimated peak of lactation)
- 56 ± 2 days post-partum (phase after weaning, basal value)

Following the protocol, all bitches underwent a general and a clinical gynaecological examination. Bitches were included into the study if no disease was reported by the owners, no signs of clinical disease were found at the initial examination, and the dogs did not receive any medication within the past 14 days prior to enrolment. Subsequent blood samplings took place at the breeders' homes to minimize stress for the bitch and the puppies and to decrease bacterial exposure during transport and visit in a clinic environment. Specific data on the dogs, their medical history and feeding were recorded on case report forms developed for this study. Owners were asked to report the time of last food intake prior to each sampling and the amount of food offered as 'x-times the baseline amount'.

Blood serum and EDTA blood were collected at all three sampling dates. Using a tourniquet, approximately 4.0 mL blood was collected into a serum tube (Kabe Labortechnik, Nürnbrecht, 4 mL Serumtube, Polystyrol) and 1.0 mL into an EDTA tube (Kabe Labortechnik, Nürnbrecht, 1 mL EDTA Tube, Polystyrol) from either the vena cephalica antebrachii or the vena saphena lateralis following the rules of good veterinary practice. All serum tubes were centrifuged within 60 min after collection for 7 min at 2000×*g* (Hettich Zentrifuge EBA 20, Hettich, Tuttlingen, Germany). Subsequently, the cooled serum (6.0–8.0°C) was sent to a commercial laboratory (IVD Institut für Veterinärmedizinische Diagnostik GmbH, Nicolaistrasse 22, 12247 Berlin—Lankwitz, Germany) via courier within 2 h. The laboratory test 'Non-Esterified Fatty Acids' (Randox; see Non-Esterified Fatty Acids|Reagents|Randox Laboratories; accessed 05/07/2022) for quantitative detection for NEFAs on Cobas Mira Plus (Roche) was used. The following parameters were analysed: red and white blood cell count, NEFA, BHB, ASAT, ALAT, GGT, GLDH, triglycerides, fructosamines. As leucocytes were expected to be elevated due to the physiological process of uterus involution post-partum, the parameter was not used as an indicator for health. If not specified different, results are given as median (IQR: 25% quantile, 75% quantile).

All statistical analyses were performed using R version 4.2.2 (R Foundation Vienna, Austria). To visualize the continuous effect of litter size and BCS on NEFA concentrations, we used a non-parametric

TABLE 1 Distribution of the bitches in breed, age, weight, parturition.

Dog nr.	Breed	Age in month d 10 ± 2 ap	Weight d 10 ± 2 ap	No of parturitions	Time since last food intake (h) d 10 ± 2 ap	Amount of feeding (times ×) d 10 ± 2 ap	BCS d 10 ± 2 ap	NEFA d 10 ± 2 ap	Leucocytes d 10 ± 2 ap
1	Cavalier King Charles Spaniel	76	9.8	5	5	5	2	1958	12.5
2	Wolfshound	84	32	4	0	5	3	1019	15.7
3	French Bulldog	28	15.3	2	0.5	1.5	3	0.624	12.3
4	Cavalier King Charles Spaniel	60	12	3	6	2	3	1676	14.0
5	Short Haired Collie	17	26	1	0.5	3	3	0.673	15.2
6	Hovawart	41	42	1	9	1.5	3	0.189	12.3
7	Giant Schnauzer	48	55	2	2	2	4	0.951	9.4
8	Miniature Schnauzer	21	11.6	1	15	2	3.5	0.638	14.2
9	Cavalier King Charles Spaniel	26	10.3	4	4	1.5	3	1295	21.5
10	Cavalier King Charles Spaniel	36	8.5	2	4	5	3	0.843	22.4
11	Wire-Haired Dachshund	23	6.4	1	2.5	5	3	0.749	16.2
12	Beagle	44	18	1	2	5	2.5	0.62	9.9
13	Hovawart	24	36	1	7	1.5	3	1104	14.2
14	Wire-Haired Dachshund	41	7.3	1	2.5	5	3.0	0.734	12.9
15	Wire-Haired Dachshund	60	7.1	3	2.5	1	3.0	0.989	12.7
16	Wire-Haired Dachshund	48	7.3	3	2.5	1	3.0	0.482	14.7
17	Swiss Mountain Dogs	41	55.6	1	4	1	4.0	0.641	11.5
18	Beagle	28	20.6	1	6.5	1	2.5	0.442	10.6
19	Dobermann	36	42	1	4	1	3.0	0.663	17.2
20	Dobermann	78	41.6	3	4	1	2.5	0.489	17.2
21	Swiss Mountain Dogs	60	56.4	1	5	0.75	4.0	1047	15.5
22	Wire-Haired Dachshund	36	6.6	1	5	1.6	3	0.866	14.5
23	Krohmfortländer	72	16.8	3	5	5	3.0	1302	10.5
24	Wire-Haired Dachshund	48	7.2	4	2	1.2	3.0	0.414	12.1
25	Short Haired Collie	20	20	1	6	4	2.5	1314	13.3
26	Wire-Haired Dachshund	68	6	2	11	2	3.0	0.554	22.8
27	Wire-Haired Dachshund	49	6.2	2	6	2	3.5	1001	10.3
28	Polish Lowland Sheepdog	72	20	3	4.5	2	3.0	0.296	11.9

(Continues)

TABLE 1 (Continued)

Dog nr.	Breed	Age in month d 10±2 ap	Weight d 10±2 ap	No of parturitions	Time since last food intake (h) d 10±2 ap	Amount of feeding (times ×) d 10±2 ap	BCS d 10±2 ap	NEFA d 10±2 ap	Leucocytes d 10±2 ap
29	Miniature Bulldog	36	5.7	2	1.5	5	3.0	0.493	14.0
30	Wire-Haired Dachshund	46	10.5	3	9.5	1.5	3.0	0.231	9.8
31	Old German herding dog	36	14.1	2	2.5	5	3	0.731	
32	Short Haired Collie	22	23	1	0	0.25	3.5	1646	11.2
33	Old German herding dog	60	22	1	1	5	3	0.685	11.9
34	Spanish Water Dog	36	23	2	0	0.75	3.5	0.64	13.0
35	Swiss Mountain Dogs	72	55.8	2	15	5	4	0.934	13.6
36	Miniature Poodle	96	14	3	2.5	3	2.5	0.865	14.1
37	American Bulldog	32	30.2	1	4	1.5	4	1034	15.4
38	Krohmfortländer	48	12.8	1	4	0.75	3	0.582	16.2

Note: On day 10(±2) ap last food intake, amount of food, Body Condition Score, NEFA concentrations, leucocytes on day 10 ±2 d ap.

'locally estimated scatterplot smoothing' (LOESS) regression. Since litter size and BCS are non-normally distributed variables, we used a non-parametric regression approach. Additionally, LOESS avoids unnecessary categorization of these continuous variables. Reference intervals (RIs) for NEFA (Doll et al., 2022) are added as dashed lines to every figure.

3 | RESULTS

Overall, 38 bitches of 20 different breeds were enrolled into the study. At the first sampling date, blood serum and EDTA blood from all dogs could be collected. On day 21±2pp, 31 bitches were available for blood sampling testing, and on day 56±2pp, 21 bitches were sampled. This led to 21 dogs, from which samples of all three time points were available. One bitch had to be excluded after the sampling as six puppies died due to a herpes virus infection. At time of enrolment, the age of the dogs was between 1.66 and 8.00 years, with a median age of 3.54 years (IQR:2.75, 5.00). The breed with the most individuals enrolled into the study was Dachshunds with nine individuals followed by Cavalier King Charles Spaniels with four individuals. All the other breeds were represented by one to three individuals. The median weight at the initial examination was 16.8 kg (IQR: 7.0, 41.9). The median NEFA concentration on day −10±2 was 0.73 mmol/L (IQR: 0.59, 1.01); on day 21±2, it was 0.57 mmol/L (IQR: 0.44, 0.82); and on day 56±2, it was 0.58 mmol/L (IQR: 0.46, 0.73) (Tables 1 and 2).

Analysing the change in NEFA concentrations between gestation and peak lactation, 26 bitches had a decrease. These bitches had median NEFA concentrations of 0.90 mmol/mL (IQR: 0.41/ 2.0) on day 10±2 ap. Yet, 10 bitches showed an increase of NEFA concentrations from late gestation to peak lactation. These dogs had a median NEFA concentration of 0.54 mmol/L (IQR: 0.20, 1.1) on day 10±2 ap. Most bitches with rising NEFA concentrations between day 10±2 ap and day 21±2 pp did not show a considerable increase towards day 56±2 pp. However, bitches with low NEFA concentrations on day 10±2 ap had an increase towards day 21±2 pp and day 56±2 pp. Three bitches had NEFA concentrations beyond the recently established RIs (Doll et al., 2022), two exceeded the RI and one fell below the RI on day 10±2 ap. Those bitches, however, had NEFA concentrations within the RIs during lactation. In total, eight bitches showed a decrease by more than 0.5 mmol/L, two bitches showed an increase by more than 0.5 mmol/L, and in 20 dogs, the NEFA concentrations did not change considerably (Figure 1).

NEFA concentrations in late gestation rose slightly with litter size (Figure 2). Litter sizes varied between 3 and 10 puppies (IQR: 5.0, 6.0, 7.8). For each puppy, the NEFA concentration on day 10±2 ap increased by 4.4%. The litter size had no effect on NEFA concentrations on day 21±2 pp and day 56±2 pp.

Data on the time from last food intake to sampling, as well as the amount of food, were documented according to the owners' declarations. Time since last food intake was in median greatest on day 10±2 ap with 4.00 h (IQR: 2.13, 5.75) followed by day 56±2

TABLE 2 Distribution of the bitches' weight, last food intake, amount of food, Body Condition Score, NEFA concentrations, leucocytes, the number of puppies alive or deceased on day 21 ± 2 pp and day 56 ± 2 pp.

Dog nr	Weight 21 ± 2 pp	Time since last food intake (h) d 21 ± 2 pp	Amount of feeding (times ×) d 21 ± 2 pp	BCS d 21 ± 2 pp	Healthy puppies 21 ± 2 pp	Puppies dead by parturition	Puppies deceased	NEFA d 21 ± 2 pp	Leucocytes d 21 ± 2 pp	Weight d 56 ± 2 pp	Time since last food intake (h) d 56 ± 2 pp	Amount of feeding (times ×) d 56 ± 2 pp	BCS d 56 ± 2 pp	Healthy puppies d 56 ± 2 pp	NEFA d 56 ± 2 pp	Leucocytes d 56 ± 2 pp
1																
2	33.0	0	5	3	4	0	0	0.523	7.0	35	0	1	3	4	0.726	10.1
3																
4	9.8	1	2.5	3.0	6	0	0	0.404	9.4							
5	21.0	0	1	4.5	7	0	0	0.574	8.1	23	0	3	3	8	0.701	13.7
6	39.0	0	2	3.0	7	0	0	0.852	12.1	40	0	1	3	7	0.765	
7	43.0	8	5	1.5	8	0	0	0.734	12.6							
8	9.8	2	2	4.5	3	1	0	0.905	10.3							
9	7.0	3	5	3.0	5	0	0	0.465	12.8	7	5	1	2.5	5	0.455	11.5
10	7.5	2	5	3.0	6	0	0	0.467	15.0	7.5	5	1	3	6	0.402	
11	4.8	2	4	3.0	5	0	0	0.219	8.9	4.8	2	1	3	5	0.474	10.1
12	15.6	3	2	2.5	6	0	0	0.857	10.8							
13	36.0	0	2	3.5	10	0	0	0.617	8.8	37	7	1	4	10	0.367	7.1
14																
15	5.8	4	3	3.0	6	0	0	0.233	9.6	5.6	2.5	2	3	6	1.08	
16	6.1	4	4	3.0	5	0	0	0.659	11.7	5.8	2.5	2	3	5	0.579	
17	45.0	3	4	4.0	2	5	0	0.379	10.7	53.3	0	2	3.5	2	0.607	6.8
18	17.3	0	5	3.0	8	0	0	0.425								
19	38.0	8	2	2.5	5	0	0	0.907	9.3	34	4	2	3	5	0.428	9.5
20	37.0	9	2	2.5	6	2	0	0.675	30.3	32	4	2	3	6	0.854	11.1
21	48.0	2	3	2.5	7	3	0	1419	7.3	49.5	4	1	3.5	7	0.964	
22	5.8	0	3	3.0	5	0	0	0.344	8.3	5	3	1	3	5	0.421	9.7
23	13.5	1	4	2.0	10	0	0	0.625	6.4	13.6	2	1.5	3	10	0.617	7.3
24	5.6	3	4	2.5	6	0	0	0.389	8.8	5.2	3	3	2.5	6	0.253	10.3
25	19.0	0	3	2.5	8	0	0	0.395	11.1							
26	5.4	0	5	2.5	6	0	0	0.476	14.7	4.9	0	2	2.5	6	0.506	12.9
27	4.2	0	5	2.5	5	0	0	0.928	13.9	4.7	0	2	3	5	0.513	11.4
28	15.9	5	1.5	3.0	4	0	0	0.495	9.1							
29	5.0	2	2	3.5	3	0	0	0.565	9.0	16	19	1	3	3	0.576	

(Continues)

TABLE 2 (Continued)

Dog nr	Weight 21 ± 2 pp	Time since last food intake (h) d 21 ± 2 pp	Amount of feeding (times x) d 21 ± 2 pp	BCS d 21 ± 2 pp	Healthy puppies 21 ± 2 pp	Puppies dead by parturition	Puppies deceased	NEFA d 21 ± 2 pp	Leucocytes d 21 ± 2 pp	Weight d 56 ± 2 pp	Time since last food intake (h) d 56 ± 2 pp	Amount of feeding (times x) d 56 ± 2 pp	BCS d 56 ± 2 pp	Healthy puppies d 56 ± 2 pp	NEFA d 56 ± 2 pp	Leucocytes d 56 ± 2 pp
30	8.5	3	3	3.0	3	0	0	0.921	8.9	7.6	9	3	3	3	0.815	6.3
31																
32	20.0	0	3	2.5	5	0	0	0.526	10.2	21.5	1	1.5	3	5	0.638	8.6
33																
34	22.0	3	2	3.0	4	1	0	0.923	10.4							
35																
36																
37																
38	11.4	0	4	3.0	8	0	0	0.674	8.9							

Note: In some bitches, the follow-up did not take place, and therefore, there is no data available (empty cells).

with median 2.5 h (IQR: 0.0, 4.0). On day 21 ± 2, the time since last food intake was only 2.0 h (IQR: 0.0, 3.0). In addition, the amount of food offered to the dogs was documented as "x-times the baseline amount". According to the owners, the maximum amount of food was fed on day 21 ± 2 pp with median 3.0-fold (IQR: 2.0, 4.0) the individual baseline food supply followed by day 10 ± 2 ap with 2.0-fold (IQR: 1.1, 5.0) and day 56 ± 2 pp with 2.5-fold (IQR: 0.0, 4.0) the baseline amount, respectively. Furthermore, haematology and clinical chemistry were analysed for the following parameters: leucocytes, fructosamine and ß-hydroxybutyrate (BHB). The mean leucocyte count was highest on day 10 ± 2 ap (IQR: 11.9, 13.6, 15.4 × 1000/µL) followed by day 21 ± 2 pp (IQR: 8.9, 9.9, 11.7 × 1000/µL) and day 56 ± 2 pp (IQR: 8.0, 10.1, 11.31000/µL). On day 10 ± 2 ap, 27 of 37 bitches underwent leucocytosis according to the RIs of 12.0 × 1000/µL given by the laboratory (Moritz et al., 2004). On day 21 ± 2, at sampling the median time since last food intake was only 2.0 h (IQR: 0.0, 3.0) .

According to Reusch et al. (1993), the upper limit for fructosamine is 374 µmol/L. None of the bitches exceeded this concentration in any sampling. Most BHB measurements showed concentrations of <0.1 mmol/L. There were only six outliers with higher concentrations on day 10 ± 2 ap as well as on day 56 ± 2 pp and 3 outliners on day 21 ± 2 pp. Median BHB concentrations were 0.13 mmol/L (IQR: 0.11, 0.14 mmol/L), 0.16 mmol/L (quartile 0.1, 0.19 mmol/L) and 0.14 mmol/L (IQR: 0.12, 0.17) at the three samplings. Delivery took place without difficulties in 26 bitches, four bitches underwent caesarean section and one bitch had assisted delivery. The usability of the body condition score is limited during gestation and lactation. However, the scores did vary only slightly as the median values at the three sampling dates were 3.0 (IQR: 3.0, 3.0), 3.0 (IQR: 2.5, 3.0) and 3.0 (IQR: 3.0, 3.0). BCS had no influence on observed NEFA values (Figure 3a–c). Disregarding two individuals, all bitches lost weight between day 10 ± 2 ap and day 21 ± 2 pp. The median weight decreased from 16.1 kg (IQR: 8.8, 29.2) on day 10 ± 2 ap to 14.6 kg (IQR: 6.3, 30.5) on day 21 ± 2 pp. In bitches with five or less suckling puppies, the median weight decreased between day 10 ± 2 ap and day 21 ± 2 pp from 10.4 kg (IQR: 6.8, 22.3) to 9.2 kg (IQR: 5.9, 21.5). In bitches with six or more suckling puppies, the median weight decreased from 19.0 kg (IQR: 9.6, 40.2 kg) to 16.5 kg (IQR: 8.5, 32.8 kg).

4 | DISCUSSION

An appropriate supply of pregnant and lactating bitches with nutrients is important for fetal development and puppy viability and health (Scantlebury et al., 2001). In that regard tools, and parameters that help to monitor the actual energy demand are helpful by practical and scientific means.

It has been suggested that dogs may show increased NEFA concentrations in situations of high-energy deficiency (Balogh et al., 2018; Doll et al., 2022). Not only in times of withheld food but also in times of greater energy demands as so during late gestation and lactation.

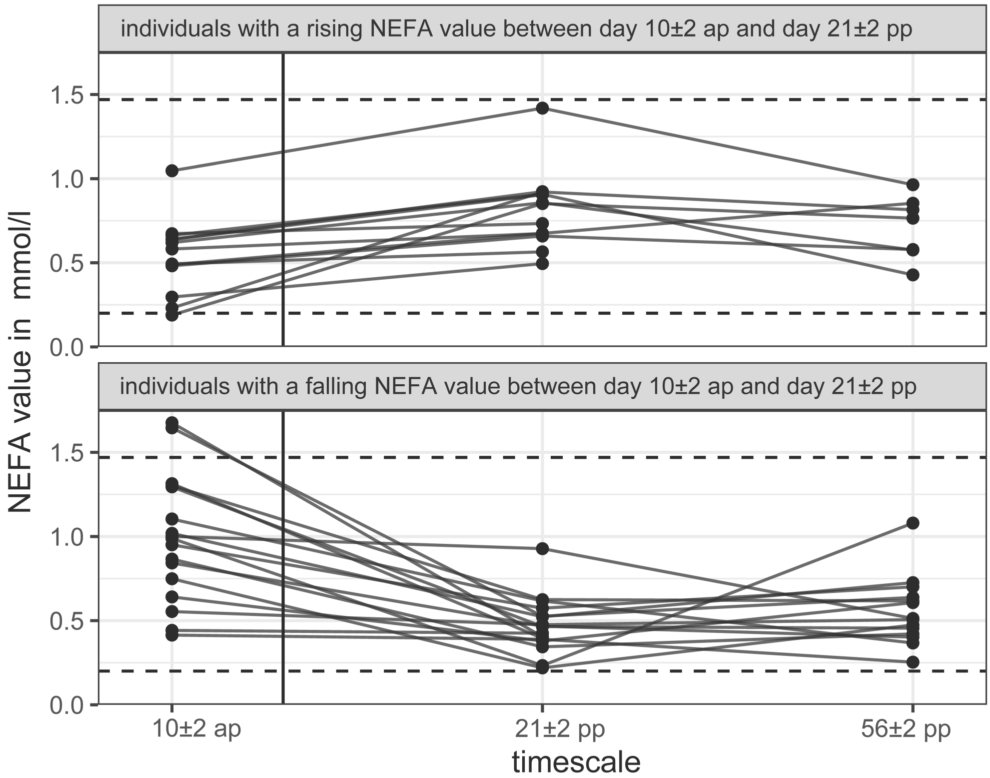

FIGURE 1 Individual blood serum NEFA concentrations at three time points in canine gestation and lactation (day 10 ± 2 ap, day 21 ± 2 pp und day 56 ± 2 pp). Dogs are divided into two groups: Bitches in the first group (n = 10) showed an increase of NEFA concentrations from day 10 ± 2 ap to day 21 ± 2 pp, the second group (n = 26) showed a decrease of NEFA concentrations from day 10 ± 2 ap to day 21 ± 2 pp. The dotted lines represent NEFA reference ranges determined for healthy male and non-pregnant female dogs (Doll et al., 2022).

Overall, our results did not reveal highly increased NEFA concentrations in late gestation or during the peak of lactation, that is, around 3 weeks after parturition. It can be hypothesized that the included dogs have received an appropriate diet meeting the demands in the respective stages of gestation and lactation. According to the owners, food supply was in many cases (28 of 38 bitches) increased by mean threefold (IQR: 2.0, 4.0) the baseline amount in peak lactation. This is in accordance with well-accepted recommendations for feeding bitches during gestation and lactation (Fontaine, 2012).

More research is necessary to assess if NEFAs are a good indicator to show undernutrition in stages such as gestation and lactation in bitches. A promising research approach would include a group of pregnant and lactating dogs which are fed standardized but inappropriate diets. In this study, privately owned breeding dogs were examined; therefore, food deprivation was impossible.

The results showed that two NEFA patterns could be observed: In one group of bitches, to which almost 75% of the bitches belonged to, the NEFA concentrations declined from late gestation towards peak of lactation. In the remaining bitches, an increase of NEFA concentrations was observed between late gestation and peak lactation.

However, these results need to be interpreted with care as the dogs belonged to different breeds, had different litter sizes and were fed individual diets by the owner. These confounders surely affected the outcome but could not be avoided due to the nature of the study approach, using privately owned dogs. Calculations pertaining to breed and offspring size in relation to NEFA concentrations would have been highly informative. However, due to the limited size of the study population, such calculations would have lacked statistical power. It can be assumed that the owners who were willing to participate in this study, in general, take great efforts to provide optimal food supply to their breeding dogs. If NEFA concentrations increase under malnutrition conditions, during disturbed pregnancies or gestation-related disorders such as progesterone mediated diabetes mellitus, remains open. The measured fructosamine concentrations indicated that none of the participating dogs suffered from diabetes.

It may have led to better insights into NEFA patterns if we would have had the opportunity to take more samples, for example during early gestation and after weaning. According to earlier research (Arlt et al., 2023), the most substantial challenge for the metabolism in lactating dogs is expected around day 21 after parturition. Therefore, we chose this time point for sampling. We were not able to collect more samples because of financial constraints and we would have expected consent problems of the dogs' owners if we would have asked for more appointments. In the recent study, we observed that several owners, initially willing to participate in the entire study, were reluctant to allow a second and third sampling, even if these samples were usually taken at the owner's homes. Some owners were concerned about potential stress for the dam and the puppies or external contamination of the puppy's environment, even if these

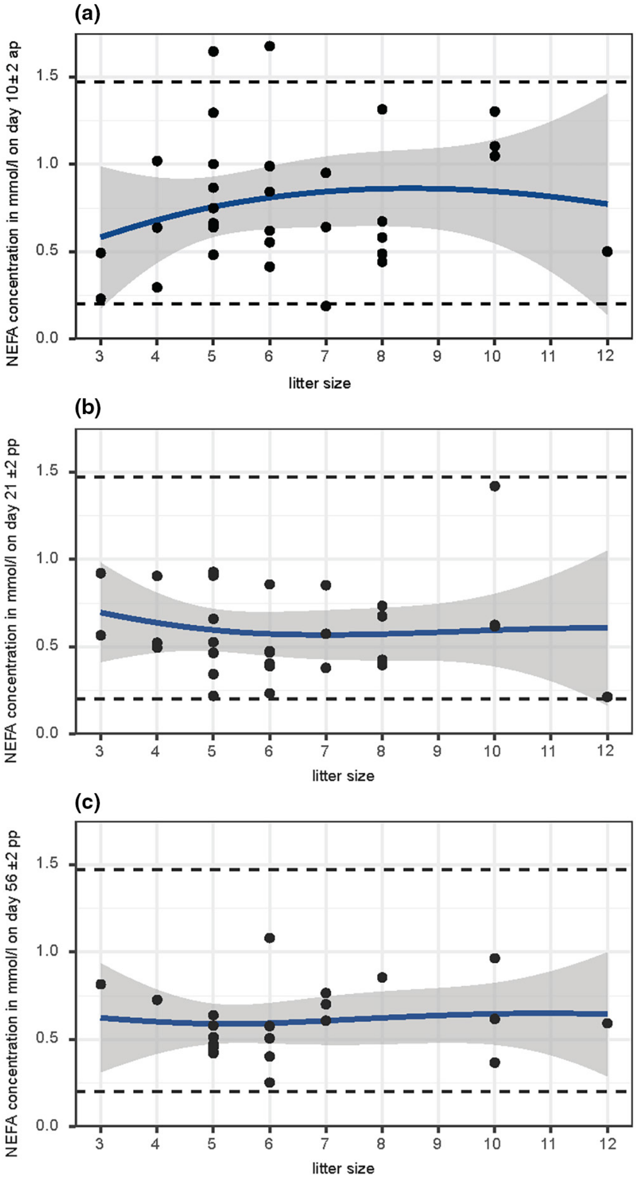

FIGURE 2 Serum NEFA concentrations in bitches on day (a)10 ± 2 ap, (b) 21 ± 2 pp and (c) 56 ± 2 pp in relation to litter size.

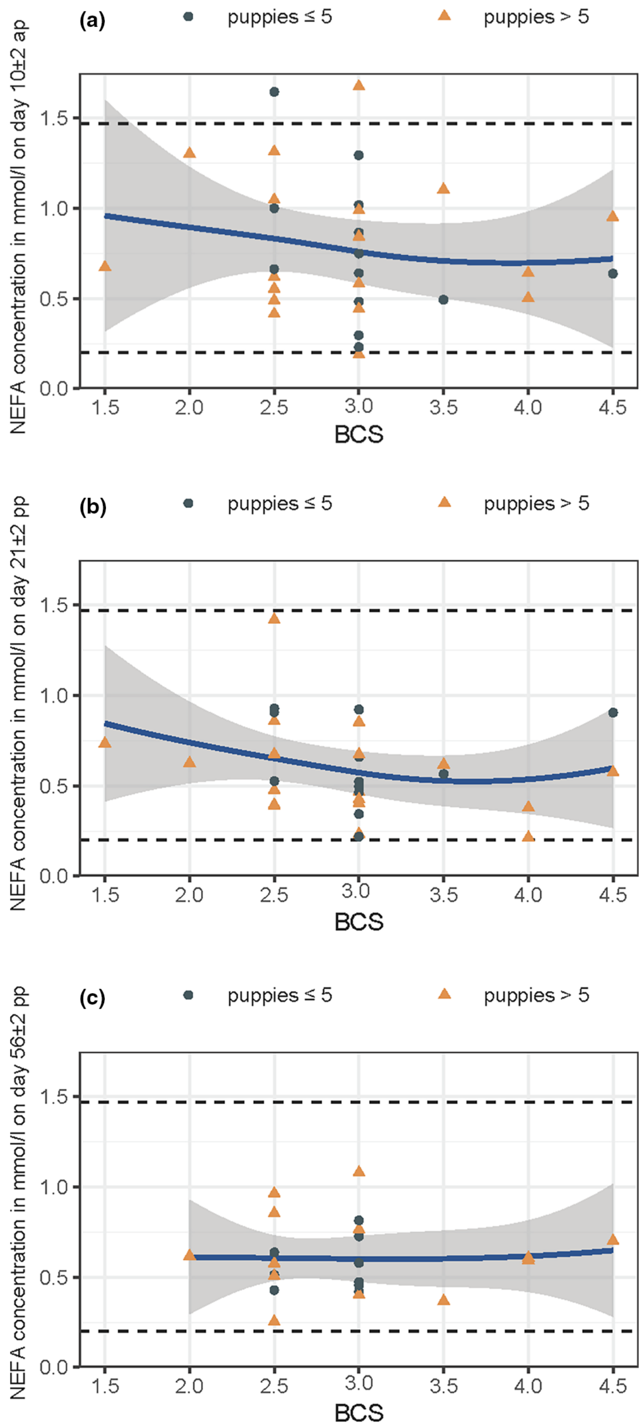

FIGURE 3 Serum NEFA concentrations in bitches on day (a)10 ± 2 ap, (b) 21 ± 2 pp and (c) 56 ± 2 pp in relation to BCS. Bitches with ≤5 puppies displayed as blue dots. Bitches with >6 puppies displayed as yellow triangles.

issues were discussed during counselling before study enrolment. One bitch was excluded after the first sampling as 6 out of 12 puppies died in the first days after parturition due to a herpes virus infection. The herpes virus infection was confirmed via PCR test.

Interestingly, litter size did affect blood NEFA concentrations in late gestation. In addition, the BHB concentrations remained below the detection limit in most bitches. However, these findings need to be interpreted with great care because of the low number of bitches with large litter sizes. Furthermore, it should be stressed that many of the owners were not aware of the number of pups expected. So, it can be supposed that knowing the actual number would have influenced the food amount. The composition, amount and quality of food provided in the different phases have not been defined and standardized, also the uptake of food by the bitches was not recorded. It can be assumed, however, that most owners cared intensively for an appropriate feeding of their bitches. Therefore, it is likely that, in the study population, no or only a small number of dogs with mal- or undernutrition are represented. In that regard, one may assume a selection bias in favour of well-nourished bitches.

To date, food supply adjustments of pregnant dogs is mainly based on information with low evidence as no large and well-designed studies have been published. In addition, some breeders adjust the food supply without a confirmation of gestation or estimation of the number of fetuses. The diagnosis of gestation can be done by relaxin measurement from around day 24 (Buff et al., 2001; Steinetz et al., 1987, 1989) by observation of abdominal extension in late gestation, ultrasound or x-ray. The number of fetuses can be assessed by ultrasound (from around day 19) (Lenard et al., 2007) or x-ray (from around day 42) (Lopate, 2008) with a sufficient accuracy. After parturition, food supply of the dam can be adjusted according to the number of the puppies born and by monitoring of weight development of the dam and the puppies (Alves, 2020; Davidson, 2003; Johnson, 2008). Besides there might be difficulties in food acceptance for both dam and puppies. In late gestation, the uterus with the growing foetuses occupies a considerable amount of space within the abdomen. Food should, therefore, be offered in many smaller portions (Ivanova & Georgiev, 2018).

An appropriate tool for monitoring if the offered and consumed food meets the energy demand of the individual bitch would be helpful to prevent hypoglycaemia in puppies. Results may be used to adjust the quantity and/or quality of food. In future studies, it should be assessed if NEFA concentrations increase in cases such as gestation loss and gestation-related diabetes. Potentially, NEFAs may be used as an indicator for early disease detection, eventually in combination with the measurement of other free fatty acids or other metabolomics parameters (Arlt et al., 2023).

5 | CONCLUSION

We conclude that NEFA concentrations widely remain within reference ranges in well-fed pregnant and lactating bitches. Nevertheless, they may be a valuable parameter to assess the actual metabolic status of malnourished pregnant and lactating bitches.

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AUTHOR CONTRIBUTIONS

S Doll, P Haimerl and S Arlt planned the study, S Doll collected the samples, S Doll, P Haimerl, A Bartel and S Arlt analyzed the data, S Doll, A Bartel and S Arlt wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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