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## Prepartum blood lead concentrations linked to subsequent cyclicity in high-producing dairy cows in a non-industrial area

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### Highlights

- Although low levels, lead (Pb) was detected in all blood samples (n=282) from 47 dairy cows in a non-industrial area.
- Blood Pb concentrations on Days 251–257 of gestation were negatively related to a subsequent return to cyclicity.
- Cows with higher Pb levels experienced a greater BCS loss during the transition period.
- Routine blood Pb tests could indicate the risk of anestrus in cows with low-grade Pb poisoning.

### Abstract

This study sought to identify the possible presence of lead (Pb) in blood and if detected to examine the relationship between blood Pb concentrations during the transition period and subsequent reproductive performance in high-producing dairy cows reared in a non-industrial area. Forty seven multiparous dairy cows were examined and/or sampled on Days 251–257 of gestation (visit 1, V1), the day of calving (V2) and on Days 8–14 (V3), 15–21 (V4), 22–28 (V5), 29–35 (V6), 36–42 (V7) and 50–56 (V8) postpartum. A mean level of  $130 \pm 17$  ppm ( $\pm$  SD) of Pb was detected in feed samples. Blood samples were collected for Pb determination from V1 to V5 and lead was present in all collected blood samples. One unit increase in blood Pb concentration in the V1 sample led to a 0.3-fold reduction ( $P=0.02$ ) in the likelihood of a cow being cyclic. Mean blood Pb concentrations were  $0.97 \pm 0.11$  and  $2.6 \pm 0.1$   $\mu\text{g/L}$  for cyclic ( $n=24$ ) and non-cyclic ( $n=23$ ) cows, respectively. Cows with a body condition score (BCS) loss of  $\geq 0.75$  units between V1 and V4 ( $n=24$ ) showed higher Pb concentrations throughout the study period than the remaining cows ( $n=23$ ;  $P < 0.001$ ). In conclusion, blood Pb levels were detected in all cows. Prepartum blood Pb concentrations were negatively related to subsequent cyclicity. Cows with higher Pb levels experienced a greater BCS loss during the transition period. Routine blood Pb tests could indicate a higher risk of anoestrus in cows with higher Pb concentrations.

Keywords: Mammals, Bovine, Heavy metals, Food pollutants, Anoestrus

### 1. Introduction

Developments in genetics and nutrition have increased milk production in dairy cows though this increase has been accompanied by a decline in reproductive performance (Lucy, 2001; López-Gatius, 2003). The transition from dry-off to early postpartum is a delicate period and poor health or well-being during this interval can affect the subsequent reproductive performance of lactating dairy cows. During the transition period, cows have to deal with reduced dry matter intake (DMI) and a negative energy balance (NEB) before they become cyclic again (López-Gatius et al., 2003; Roche et al., 2009). Thus, any external factor that negatively affects animals during the transition period will have serious repercussions on dairy economy.

Heavy metals occur naturally in the environment though human activities such as industry have led to their increased concentrations in air, crops and water. Lead (Pb) is an ubiquitous heavy metal with known negative effects such as inducing reactive oxygen species (ROS) which in turn, increases lipid peroxidation (Upasani et al., 2001). This pollutant is considered furthermore an important reproductive toxic chemical in humans (Kumar and Mishra, 2010; Buck Louis, 2014). For example, there is evidence indicating that lead may be behind up to 5% of the cases of unexplained human male infertility (Benoff et al., 2003). Plants may bio-accumulate this toxic metal and when grazing animals consume such plants it enters the food chain with possible impacts on both animal and human health (Miranda et al., 2005; Aslani et al., 2012). Lead poisoning is more common in farm ruminants than wild ones (Aslani et al., 2012). In farm animals, a blood Pb concentration of up to 0.25 µg/mL is considered safe, whereas levels in excess of 0.35 µg/mL can be toxic (Radostitis et al., 2000). Recently, chronic exposure to low Pb levels has been linked to high blood Pb concentrations in cows reared in an industrial area (Mohajeri et al., 2014). However, to our knowledge, no study has examined the possible clinical effects of low (considered non-toxic) blood Pb levels on reproductive variables in dairy cows. The present study was performed in high-producing dairy cows reared in a non-industrial area of Spain. Its aims were: (1) to identify the possible presence of Pb in food and blood and (2) to examine the possible relationship between blood Pb concentrations during the transition period and subsequent reproductive performance.

## **2. Material and methods**

### **2.1. Cattle and herd management**

The study was performed from March 2014 to January 2015 on a single commercial Holstein-Friesian dairy herd in northeastern Spain comprising 820 cows. The farm was located in an agricultural area without industries 25 km around. Mean annual milk production and culling rates for the study period were 11,940 kg and 27%, respectively. Herd management included housing in free stalls with cubicles with concrete slatted floors, and the use of fans and water sprinklers in the warm season. The herd was subjected to a reproductive health programme including meticulous postpartum checks. The cows calved all year round, were milked three times daily and were fed complete rations. Feeds consisted of cottonseed hulls, barley, corn, soybean, and bran. Roughage was mainly provided as corn but also as barley or alfalfa silage and alfalfa hay. Rations were in line with NRC recommendations (National Research Council, 2001). Presence of mycotoxins (aflatoxin B1, deoxynivalenol, zearalenone, ochratoxin A and T-2 toxin) was determined monthly in each feed and in the complete ration given to the cows. No samples exceeded the maximum level established in the EU for aflatoxin B1 (Commission Regulation 574/2011 amending Annex I to Directive 2002/32/EC 2011), neither the recommended values of the other mycotoxins tested (European Commission, 2006, 2011, 2013a).

All animals were tuberculosis and brucellosis free as indicated by yearly tests from 1985 to 2015. Vaccination programmes for the prevention of bovine viral diarrhoea (BVD) and infectious bovine rhinotracheitis (IBR) included modified live vaccines (Cattlemaster, Pfizer, New York, USA) for animals 6–8 months old. Pregnant animals were given killed vaccines (Triangle 4, Boehringer Ingelheim, Barcelona, Spain) during the 7th month of each gestation period. Parous cows that were not pregnant on Day 150 postpartum received a further killed vaccine.

### **2.2. Reproductive health management**

Dry cows were kept in a separate group and transferred to a “calving group” 7–25 days before delivery depending on their body condition score (BCS) (López-Gatius et al., 2006) and whether they were carrying twins (López-Gatius and Garcia-Ispuerto, 2010). An early postpartum, or “fresh cow” group was established for postpartum daily checks and nutrition tests. At 7–20 day postpartum, primiparous and multiparous lactating cows were transferred to separate groups. In the postpartum checks, the following puerperal diseases were treated until resolved or until culling: signs of injury to the genital area (i.e., vaginal or recto-vulvar lacerations), metabolic diseases such as hypocalcemia and ketosis (the latter, diagnosed during the first or second week postpartum), retained placenta (foetal membranes retained

longer than 12 h after parturition), or primary metritis (acute puerperal metritis diagnosed during the first or second week postpartum in cows not suffering placental retention).

The herd was maintained on a weekly reproductive health programme. This involved examining the reproductive tract of each animal by ultrasound from 15 to 21 and 50–56 days postpartum to check for normal uterine involution and ovarian structures. The entire reproductive tract was examined by ultrasound using a portable B-mode ultrasound scanner (Easy-Scan with a 7.5 MHz transducer). Scanning was performed carefully and slowly along the dorsal/lateral surface of the cervix and each horn and then the ovaries. Cranial cervical size and endometrial thickness were measured using the internal calipers of the ultrasonographer. Reproductive disorders diagnosed at this time such as endometritis or ovarian cysts were treated on a weekly basis until resolved. Cows were classed as suffering endometritis according to the following criteria: the presence of echogenic intrauterine fluid, cervical diameter  $\geq 4$  cm, or endometrial thickness  $\geq 0.75$  cm (López-Helguera et al., 2012). An ovarian cyst was diagnosed when a follicular structure larger than 20 mm in diameter (external diameter including the wall) was detected in either one or both ovaries in the absence of a corpus luteum (CL) and uterine tone (Hanzen et al., 2007). The presence of a CL in one or both ovaries was also recorded.

All postpartum reproductive disorders were resolved before 50 days in milk. Since a retained placenta or puerperal metritis were previously related to subsequent pregnancy loss in cows (López-Gatius et al., 1996), both disorders were always treated by introducing oxytetracycline boluses into the uterus plus cefquinome sulphate i.m. and prostaglandin F<sub>2</sub> $\alpha$  at the end of treatment. Prostaglandin F<sub>2</sub> $\alpha$  or a synthetic analogue was also used to treat endometritis and ovarian cysts. In the latter case, treatment was subsequent to manual rupture of the cystic structure per rectum (Hanzen et al., 2008).

Cows 60 days in milk and not detected to be in oestrus in the preceding 21 days were examined weekly by ultrasound until oestrus following a 5-day progesterone-based treatment (García-Ispuerto and López-Gatius, 2014) or until artificial insemination (AI) was performed during a spontaneous oestrus. All cows were artificially inseminated. Although oestrus detection using pedometers started on day 14 postpartum (López-Gatius et al., 2005), the voluntary waiting period for the herd was 50 days.

Over the study period, means ( $\pm$ SD) for the interval parturition-first insemination, milk production at 50 days postpartum and lactation number were  $69\pm 11$  days (51–97 days),  $45\pm 9.8$  kg (25–72 kg) and  $5\pm 1$  lactations (4–9 lactations), respectively. Rates of placenta retention, endometritis, cyclicity and conception at first AI were 38.3%, 23.4%, 51.1% and 40.4%, respectively. Oestrus was recorded at least one time in 22 (91.7%) of the 24 cyclic cows, whereas non-cyclic cows did not show oestrus signs.

### 2.3. Insemination and pregnancy diagnosis

Oestrus was confirmed by palpation per rectum (López-Gatius and Camón-Urgel, 1988, 1991) in cows deemed to be in oestrus using the pedometer system and the animals inseminated at this time. Only cows showing oestrus signs with strong uterine contractility (determined by uterine tone) and copious, transparent vaginal fluid were inseminated (Roelofs et al., 2010; López-Gatius, 2012). If cows returned to oestrus, their status was confirmed by examination per rectum, and the animals were recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 days post-insemination. Cows diagnosed as non-pregnant were either returned to the reproductive programme or scheduled for culling.

### 3. Experimental design

Trace and heavy metals such as Cu, Zn, Mn, Se, Cd and Pb were analysed in food samples in three different herds. Since only Pb was present in all samples, possible effects of Pb was analysed in a herd with more reproductive disorders registered per cow. Based on the hypothesis that small amounts of Pb may bio-accumulate in living organisms, only multiparous cows with at least three finished lactation periods were included in the study. Forty seven cows were examined and/or sampled in weekly visits (except at parturition) on Days 251–257 of gestation (visit (V) 1), on the day of calving (V2) and on Days

8–14 (V3), 15–21 (V4), 22–28 (V5) 29–35 (V6), 36–42 (V7) and 50–56 (V8) postpartum. Only cows delivering singletons were included in the study. Although blood samples were collected from all prepartum cows, since placenta retention has been related to most postpartum reproductive disorders (Labèrnia et al., 1998), for each cow suffering placenta retention two healthy cows entered in the study in order to determine the possible implication of Pb in this pathology. No cows suffering other reproductive or clinical disorders were included in the study. Criteria of exclusion were cows suffering dystocia, ketosis, puerperal metritis, lameness, mastitis or digestive disorders. Blood samples were collected for Pb determinations from V1 to V5 from the coccygeal vein into EDTA vacuum tubes (BD Vacutainer™, Becton-Dickenson and Company, Plymouth, UK). Tubes including whole blood were stored at  $-20^{\circ}\text{C}$  until analysis. On visits V1 and V4, BCS was recorded using a 5 point scale (López-Gatiús et al., 2003). Uterine contents and structures and ovarian structures were recorded from V4 to V8 by ultrasound. All exams were performed by the same veterinarian.

Three feed and three water samples were collected monthly during the study period. Sampling followed official normalized procedures. Each aggregate feed sample weighed approximately 500 g and was collected according to the procedures described in the European Commission Regulation (2013b). Sample containers were sealed, labelled and stored at refrigeration temperature until analysed in the laboratory.

#### **4. Blood Pb assays**

##### **4.1. Sample pretreatment**

In all blood samples, total metal concentrations were quantified by inductively coupled argon plasma mass spectrometry (ICP-MS) following their dilution 1:10 in a basic diluent composed of 2% (w/v) 1-butanol (Sigma-Aldrich, Steinheim, Germany), 0.05% (w/v) EDTA (Fluka, Steinheim, Germany), 0.05% (w/v) Triton X-100 (Sigma, Steinheim, Germany), 1% (w/v)  $\text{NH}_4\text{OH}$  (Sigma-Aldrich, Steinheim, Germany) and Milli-Q water ( $\geq 18.2\text{ M}\Omega\text{ cm}$ ) (Gajek et al., 2013).

Feed samples (0.5 g) were digested with 3 mL 67% (w/w)  $\text{HNO}_3$  TraceMetal Grade (Fisher Scientific, Leicestershire, UK) and 2 mL 30% (w/v)  $\text{H}_2\text{O}_2$  p.a. (Panreac, Barcelona, Spain) (EN-13805:2002) in acid-pretreated TFM vessels with a microwave system (Milestone 1200, Bergamo, Italy) according to the following heating program: 1) 1 min at 250 W, 2) 3 min at 0 W, 3) 4 min at 250 W, 4) 4 min at 400 W, 5) 3 min at 600 W. After cooling to room temperature, the digested samples were weighed, diluted to 25 mL using Milli-Q water, and stored at  $4^{\circ}\text{C}$ .

##### **4.2. Sample analysis by ICP-MS**

Total lead was quantified using a 7700x ICP-MS (Agilent Technologies, Inc, Tokyo, Japan) with Ni sampler and skimmer cones, a MicroMist glass concentric nebulizer and a He collision cell. The operating conditions were as follows: RF power 1550 W, carrier gas flow rate  $1.01\text{ L min}^{-1}$ , helium collision gas flow rate,  $4.3\text{ mL min}^{-1}$ , spray chamber temperature  $2.0^{\circ}\text{C}$ , sample depth 10.0 mm, nebulizer pump 0.1 rps, extract lens 1 voltage 0.0 V and extract lens 2 voltage  $-1.95.0\text{ V}$ . The monitored isotope was  $^{208}\text{Pb}$  and on-line internal standard  $^{209}\text{Bi}$ .

Appropriate amounts of standard stock solutions containing  $1000\text{ mg L}^{-1}$  in 2% nitric acid of each element (High Purity Standards) were mixed to prepare standard solutions. These were then diluted with 1%  $\text{HNO}_3$  and 0.5%  $\text{HCl}$  (in the standards for feed samples) or with the basic blood diluent (in the standards for blood samples) at five concentration levels, and then used to construct calibration curves. Calibration curves showed a linear response across this range with a correlation coefficient  $\geq 0.999$ . The limit of detection (LOD) for Pb was  $0.003\text{ }\mu\text{g/L}$ , calculated as  $3 \times \text{S.D.} / \text{slope}$  of the calibration curve, where SD is the standard deviation of the response of ten blanks (Currie, 1995). All samples were analysed in batches with blanks and known standards. All blanks, standards, and samples were analysed in triplicate.

##### **4.3. Data collection and analysis**

The following data were recorded for each animal: calving date, lactation number, blood Pb concentrations from V1 to V5, retention of the placenta, endometritis or ovarian cysts detected in V4, presence of at least a corpus luteum from V4 to V8, oestrus recorded from Day 21 postpartum, BCS loss (BCS in V1 minus BCS in V4), milk production at 50 days postpartum (mean production during the three days before day 50) (low producers <40 kg versus high producers  $\geq$ 40 kg) and oestrus at the first AI (spontaneous vs. synchronized), semen providing bull, AI technician and pregnancy diagnosis 28–34 days post-AI. Cows with no oestrous signs detected in the 21 days preceding V8 and with no luteal tissue detected from V4 to V8 were recorded as non-cyclic and the remaining cows as cyclic. In our geographical region, there are only two clearly differentiated weather periods: warm (May to September) and cool (October to April) (Labèrnia et al., 1998; Garcia-Ispierto et al., 2007). Calving and AI dates were used to analyse the effects of season (cool vs. warm) on the pregnancy rate.

All statistics procedures were performed using the SPSS package version 18.0 (SPSS Inc., Chicago, IL, USA) with significance set at  $P < 0.05$ . Four binary logistic regression models were built entering placenta retention, endometritis, cyclicality and positive pregnancy diagnosis 28–34 days following first AI as the dependent variable. Independent factors for each dependent variable were: calving season, lactation number, and blood Pb concentrations from V1 to V5 for retention of placenta; calving season, lactation number, blood Pb concentrations from V1 to V5, retention of placenta and BCS loss for endometritis; calving season, lactation number, milk production at 50 days postpartum, blood Pb concentrations from V1 to V5, retention of placenta, BCS loss, ovarian cysts and endometritis for cyclicality; calving and AI season, lactation number, milk production at 50 days postpartum, blood Pb concentrations from V1 to V5, retention of placenta, BCS loss, ovarian cysts, endometritis and cyclicality for positive pregnancy diagnosis 28–34 days following first AI.

The effects of the variables calving season, lactation number, milk production at 50 days postpartum, retention of placenta, BCS loss, ovarian cysts, endometritis and cyclicality on blood Pb concentrations for all animals were assessed by GLM repeated measures analyses of variance.

## 5. Results

A mean level of  $130 \pm 17$  ppm ( $\pm$  SD) of Pb was detected in the feed samples. No Pb was detected in water during the study period. Lead was detected in all collected blood samples with mean values of  $2.8 \pm 1.1$   $\mu\text{g/L}$ , ranging from 0.9 to 17.0  $\mu\text{g/L}$ . Cows experiencing a BCS loss  $\geq 0.75$  units showed a mean concentration of Pb of  $2.93 \pm 1.2$   $\mu\text{g/L}$ , compared to the  $2.15 \pm 1.0$   $\mu\text{g/L}$  of cows that lost less than 0.75 units. Major BCS losers (2 units) had a mean Pb concentration of  $6.89 \pm 2.5$   $\mu\text{g/L}$ .

Fig. 1 provides descriptive values for blood Pb concentrations by BCS loss. Cows experiencing a BCS loss  $\geq 0.75$  units ( $n=23$ ; 49.9%) showed higher Pb concentrations throughout the study period than the remaining cows (between subject effect;  $P < 0.001$ ). The remaining factors analysed were unaffected by blood Pb concentrations.

Based on the odds ratio, a one unit increase in blood Pb concentration in V1 led to a 0.3-fold reduction (95% confidence interval: 0.1–0.8;  $P=0.02$ ) in the likelihood of cyclicality. Blood concentrations of Pb were  $0.97 \pm 0.11$  and  $2.6 \pm 0.1$   $\mu\text{g/L}$  for cyclic and non-cyclic cows, respectively. No significant interactions were found.

No significant effects were observed of any of the variables examined on placenta retention, endometritis and a positive pregnancy diagnosis 28–34 days following first AI.

## 6. Discussion

As far as we are aware, this is the first study to correlate the presence of blood Pb with anestrus in high producing dairy cows in a non Pb-polluted area. Cows were fed with Pb-contaminated food and although the blood Pb concentrations observed here may be considered non-toxic, Pb was detected in all animals. Effectively, the mean Pb concentrations of 2.8  $\mu\text{g/L}$  found in this study were far below the toxic

concentrations of 350 µg/mL described by Radostitis et al. (2000). Other authors have described the acute poisoning effects of higher concentrations of Pb in cows living in polluted areas (Dey et al., 1997; Swarup et al., 2007; Mohajeri et al., 2014). It is well known that Pb has a bio-accumulative, non-beneficial effect in mammals and that it is potentially harmful even at low concentrations (Mcintyre and Mills, 1975). Thus, it is possible that the blood concentrations detected in this study could have a subclinical chronic effect. Since only old cows were included in our study, in future studies, Pb levels need to be determined in young animals.

Lead is a major environmental toxin that causes haematological, gastrointestinal, and neurological dysfunction in mammals. The toxic effects of Pb are mainly produced through its oxidative effects that deplete glutathione and protein-bound sulfhydryl groups, in turn, promoting the production of ROS as superoxide ions, hydrogen peroxide, and hydroxyl radicals (Stohs and Bagchi, 1995). One of the most critical moments in the life of a dairy cow is the transition period (Goff and Horst, 1997) when the capacity of antioxidant defences is exceeded by the production of ROS (Lykkesfeldt and Svendsen, 2007). Oxidative stress plays a key role in several pathological conditions related to animal reproduction (Lykkesfeldt and Svendsen, 2007; Combelles et al., 2009). Thus, in cows already suffering oxidative stress during the peripartum period, the presence of Pb could impair reproductive functions such as their return to cyclicity because oxidative stress decrease neutrophil function. The question that arises is why cows showing placenta retention or endometritis did not have more blood Pb concentration than the remaining animals. Moreover, the stress that high producing dairy cow suffers during the transitional period, can also condition the results found in the study.

Remarkably, cows experiencing a greater BCS loss than 0.75 in the peripartum period had higher blood Pb levels than the remaining animals. Lead has been described to show an appetite-depressant effect (Hammond and Succop, 1995) that can decrease feed consumption. It is thus tempting to speculate that cows with greater amounts of Pb will have to mobilize more of their own reserves during the delicate peripartum period and thus suffer a greater BCS loss than the remaining animals. We also propose that besides the direct effects of Pb on reproduction, a consequent loss in BCS will have further clinical impacts (López-Gatius et al., 2003).

Bone is considered the best marker of lead exposure (Bellis et al., 2012) and this type of sample, and probably other tissues, will provide more accurate Pb determinations than whole blood (de Figueredo et al., 2014), though animal sacrifice is required. BCS loss is only a marker of mobilization of fat, and other substances like lead. The subclinical toxicity of Pb in high producing dairy cows deserves further attention to determine the real implications of this findings.

## **7. Conclusions**

Prepartum blood Pb concentrations could affect the subsequent return to cyclicity of dairy cows in a non-industrial area. Cows with greater blood Pb concentrations during the peripartum period suffered a greater BCS loss. These results indicate that routine blood testing for Pb during the prepartum period could help to predict the risk of anoestrus in cows suffering low-grade chronic Pb exposure.

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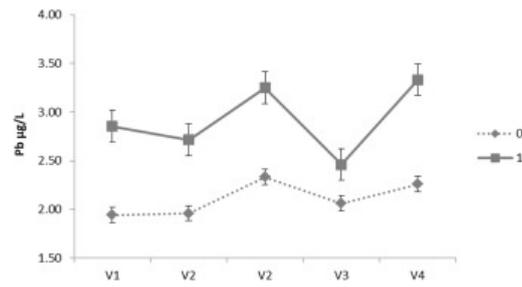


Fig. 1. Mean blood lead (Pb) concentrations recorded on Days 251–257 of gestation (V1), the day of calving (V2) and on Days 8–14 (V3), 15–21 (V4), and 22–28 (V5) postpartum (mean±SE) in lactating dairy cows (n=47) according to their body condition score loss (0: less than 0.75 units, n=23; 1:  $\geq$ 0.75 units, n=24) (between subject effect;  $P < 0.001$ ).

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