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Modulation of Aryl Hydrocarbon Receptor Target Genes in Circulating Lymphocytes from Dairy Cows Bred in a Dioxin-like PCBs Contaminated Area

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Abbreviations: PCDDs, polychlorodibenzo-*p*-dioxins; PCDFs, polychlorodibenzofurans;
PCBs, polychlorobiphenyls; DL, dioxin-like; AHR, aryl hydrocarbon receptor; ARNT, AHR
nuclear translocator; XREs, xenobiotic-response elements; AHRR, AHR repressor; TCDD,
2,3,7,8 tetrachloro-dibenzo-*p*-dioxin; TEQ, toxic equivalent; DMSO, dimethylsulfoxide;
qRT-PCR, quantitative reverse transcription PCR; cDNA, complementary DNA; CNRQ,

calibrated normalized relative quantity; FC, fold-change; WHO, World Health Organization;
EROD, 7-ethoxyresorufin O-deethylase.

ABSTRACT

Animal productions (i.e. fish, eggs, milk and dairy products) represent the major source of exposure to dioxins, furans, and dioxin-like (DL) polychlorobiphenyls for humans. The negative effects of these highly toxic and persistent pollutants are mediated by the activation of the Aryl Hydrocarbon Receptor (AHR) that elicits the transcriptional induction of several genes, including those involved in xenobiotic metabolism. Previously we demonstrated the presence and functioning of the AHR signaling pathway in primary cultures of bovine blood lymphocytes. The aim of the present study was to investigate by real time PCR the expression and the inducibility of selected target genes (i.e. AHR, AHR nuclear translocator (ARNT), AHR repressor, CYP1A1 and CYP1B1) in uncultured cells from dairy cows naturally exposed to DL-compounds. The study was carried out on two groups of animals bred in a highly polluted area and characterized by a different degree of contamination, as assessed by bulk milk TEQ values, and a control group reared in an industry free area. Bovine lymphocytes expressed only AHR, ARNT and CYP1B1 genes to a detectable level; moreover, only CYP1B1 expression appeared to be correlated to TEQ values, being higher in the most contaminated group, and decreasing along with animal decontamination. Finally, lymphocytes from exposed cows displayed a lower inducibility of both CYP1A1 and CYP1B1 after the *in vitro* treatment with a specific AHR ligand. In conclusion, our results indicate that DL-compound contaminated cows may display significant changes in AHR-target genes expression of circulating lymphocytes.

Key Words: Dioxins; Dioxin-like PCBs; Circulating Lymphocytes; Bovine; Aryl Hydrocarbon Receptor; Gene Expression; CYP1B1

1. Introduction

Polychlorodibenzo-*p*-dioxins (PCDDs), polychlorodibenzofurans (PCDFs) and polychlorobiphenyls (PCBs) are persistent environmental pollutants of main anthropogenic origin. A wide array of pathological effects has been reported following the exposure to such contaminants, including cancer and developmental defects, as well as perturbation of the endocrine, reproductive, and immune systems (Mandal, 2005). Only a relatively small number of congeners, collectively referred to as dioxin-like (DL), displays the property to bind with different affinities to the cytosolic aryl hydrocarbon receptor (AHR), which is considered a key event in DL-compound toxicity (Denison et al., 2011). After translocating into the nucleus, the complex first binds to the AHR nuclear translocator (ARNT) protein and subsequently to xenobiotic-response elements (XREs), resulting in the transactivation of a number of target genes encoding for biotransformation enzymes (e.g. CYP1A1, CYP1A2, CYP1B1, UGT1A, GSTA1, and NQO1), for the AHR repressor (AHRR) regulating the expression of AHR itself, and for proteins involved in cell growth, death, and migration (Hahn et al., 2009).

The AHR-dependent pathway appears to be well conserved across species and widely expressed not only in liver, but also in several extrahepatic tissues and cell types, such as skin, spleen and lymphoid tissues, lung, ovary, testis, and prostate (Harper et al., 2006; Mason and Okey, 1982). In recent years, a considerable body of knowledge has accumulated about the presence of the AHR pathway in circulating blood cells, and namely in peripheral lymphocytes. In particular, the constitutive expression of target genes such as CYP1A1 and CYP1B1, and their positive modulation brought about by several AHR ligands, including DL-compounds, has been reported in mouse, rat, and human lymphocytes under both *in vitro* and *in vivo* conditions (Nohara et al., 2006). More to the point, CYP gene expression comparable

to that found in liver was detected in human blood mononuclear cells (Furukawa et al., 2004) and quantitative evidence for similarities in the AHR-mediated CYP regulation suggest that circulating lymphocytes could serve as a surrogate for studying AHR-dependent changes in tissue CYP expression (Saurabh et al., 2010).

Owing to their high lipophilicity and to a general resistance to CYP-mediated biotransformation, DL-compounds tend to accumulate in the food chain being stored in fatty tissues of animals and humans and excreted in dairy milk and eggs. Food of animal origin is generally the principal route of human exposure to such pollutants, cow's milk and dairy products, bovine and porcine fats, eggs, and fish being identified as the main contributors (Bernard et al., 1999; Larsen, 2006). The monitoring of food producing species exposure to DL-compounds is therefore a critical step in the risk management procedures. Indeed, in the last decade the accidental contamination of certain feed ingredients has been the cause of veritable alimentary crises as was the case for the Belgian (1999), Irish (2008), and German (2010) incidents (Casey et al., 2010; Kupferschmidt, 2011). Episodes of more limited extent have been also recently recorded in Italy and France, as the result of either the pollution of pastures due to the emissions of certain industrial settings, or the illegal practice of dumping or burning solid wastes releasing PCDD-, PCDF-, and DL-PCBs (Diletti et al., 2004; Marchand et al., 2010).

Analytical methods for screening and confirmation of DL-compounds in animal productions are rather expensive and time consuming, thereby limiting the number of tests to be carried out in the frame of the National Control Plans on food and feed, as well as their usefulness in case of contamination outbreaks. A number of different approaches are therefore being developed, including bioassays like CALUX test (Denison et al., 2004) or devices based on a biological response such as biosensors (Chobtang et al., 2011). Landi and coworkers (Landi et al., 2003) reported on differences in AHR-dependent gene expression

and inducibility in circulating lymphocytes from individuals exposed to 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD) during Seveso's accident occurring 20 years before, making therefore peripheral leukocytes potential candidates for use as easily accessible biomarkers of exposure to DL-compounds in living organisms. As regards cattle, while the constitutive expression and the ligand-mediated modulation of the AHR pathway has been recently characterized in cultured blood lymphocytes (Girolami et al., 2011), no data pertaining living animals are available on this topic. This study was undertaken to gain further insight into the regulation of the AHR signaling pathway in circulating lymphocytes of dairy cows involved in a recent episode of DL-compound contamination occurring in the Susa Valley (northern Italy). Cows had been fed contaminated forage and hay collected nearby a high-temperature steel production plant, resulting in unacceptable levels of PCDD/Fs and DL-PCBs in bulk milk. An increased chromosomal fragility (Di Meo et al., 2011) and plasma changes suggestive of a marked oxidative stress (Spagnuolo et al., 2012) were also reported in the cows subject of the present investigation.

2. Materials and methods

2.1 Reagents

Histopaque-1077, dimethylsulfoxide (DMSO), and all cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). PCB126 was supplied by LabService Analytica S.r.l. (Anzola Emilia, BO, Italy). All the materials for the quantitative (q) reverse transcription (RT) PCR analysis (including RNA extraction) were from Qiagen (Valencia, CA).

2.2 Animals and sampling

The study was performed in the Susa Valley (northern Italy) on 60 dairy cows, 40 of which came from two different farms (A and B, 20 animals each) located near a DL-compound contaminated area; the remaining 20 animals, reared in a non-contaminated area of the same valley, were used as controls (C). Cows from farms A and B were mainly Piedmontese X Valdostana cross-breeds, while control animals were Valdostana breed. The housing conditions were similar in all the farms. The animals were kept inside during the cold season (4 to 5 months/year), being offered hay and unifeed, while for the rest of the year cows were allowed to graze on pastures on areas that for farms A and B were located nearby the source of contamination. The extent of the DL-compound exposure was defined by measuring TEQ values of PCDDs, PCDFs, and DL-PCBs in bulk milk samples collected by the Regional Veterinary Services. The official chemical analyses were carried out by Istituto Zooprofilattico Sperimentale of Piemonte, Liguria, e Valle d'Aosta using a validated High Resolution Gas Chromatography Mass Spectrometry method. Milk sampling from the contaminated farms was performed twice at an 8-month interval (sampling I and II, respectively), in order to monitor the decrease of animal contamination, which was achieved by bringing the cows on less contaminated pastures and providing non-contaminated hay even during the outdoor season. TEQ values are summarized in Table 1.

Peripheral venous blood from each animal was collected by jugular venipuncture in EDTA tubes approximately 2 months after milk sampling I and II, respectively. In particular, blood sampling I was performed in April, and blood sampling II was performed in January. Due to farm management problems, blood sampling II was performed on 18 cows from farm A, 19 from farm B, and only 12 from farm C. Three months after blood sampling II, an additional lymphocyte collection was performed from a selected number of the already sampled cows (11 from farm A, and 11 from farm C) to establish primary cultures and to assess their *in vitro* inducibility by DL-compounds, as reported by Girolami et al. (2011).

2.3 Blood lymphocyte isolation, culture and treatment

Peripheral blood lymphocytes were isolated by Histopaque-1077 gradient centrifugation, using the method described by Spalenza et al. (2011). In certain experiments, lymphocytes were counted with a hemocytometer and viability was assessed with the trypan blue exclusion test (> 90%). Cells were seeded at 2×10^6 cells/ml into 10-cm dishes and cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated horse serum, 2mM L-glutamine and 10 μ g/ml phytohemagglutinin, at 37°C and 5% CO₂. After 2 h of pre-incubation, lymphocytes from each animal were treated with the solvent (DMSO) alone (used as control) or with PCB126 100nM, and lysed after 2 h. Although the EC50 for CYP1A1 and CYP1B1, the two most responsive AHR-target genes, has been reported to be 1.73nM and 0.83nM, respectively, such concentration was used since it was found to produce the maximal induction rate (Girolami et al., 2011).

2.4 RNA extraction

Total RNA, both from uncultured and cultured cells, was isolated using RNeasy Mini Kit, according to the manufacturer's protocol. RNA purity and quantity was evaluated by absorbance readings using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Illkirch Cedex, France). The ratio of the optical densities measured at 260 and 280 nm were > 1.9 for all RNA samples. RNA quality was assessed by the RNA 6000 pico LabChip Kit in the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). All the samples had a RNA Integrity Number (RIN) > 7.

2.5 Quantitative reverse transcription PCR

One μg of total RNA was reverse transcribed into complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions, in a final volume of 20 μl . The cDNA was subsequently diluted in nuclease-free water and stored at -20°C . Sufficient cDNA was prepared in a single run to perform all the q-PCR experiments. The expression of CYP1A1, CYP1B1, AHR, ARNT, and AHRR was measured in uncultured lymphocytes, while in cultured lymphocytes the expression of CYP1A1 and CYP1B1 was evaluated. Primers for AHR, ARNT, AHRR, CYP1A1, CYP1B1 and GAPDH were from Girolami et al. (2011), whereas primers for PPIA and S24 were from Spalenza et al. (2011). q-PCR reactions were performed on 100-900 ng of cDNA, in a final volume of 25 μl consisting of the 1 X QuantiFast SYBR Green PCR Master Mix and an optimized concentration of each primer set (300-900 nM range). PCR amplification was run on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using 96-well optical plates under the following conditions: 5 min at 95°C for polymerase activation, and 40 cycles of 15 s at 95°C and 60 s at 60°C . Each reaction was run in triplicate, and a no-template control was included using water instead of cDNA.

2.6 Data analysis

For uncultured lymphocytes, the relative gene expression was calculated as described by Hellemans et al. (2007), using S24 and PPIA as reference genes (Spalenza et al., 2011) and RNA from bovine liver as an inter-run calibrator. Data were expressed as calibrated normalized relative quantity (CNRQ). Statistical significance was determined by the Kruskal-Wallis ANOVA test followed by the Dunn's multiple comparisons test (sampling I) or by the Wilcoxon Signed-Rank Test (sampling II).

For cultured lymphocytes, induction of CYP1A1 and CYP1B1 expression was calculated with the $2^{-\Delta\Delta\text{Ct}}$ method using GAPDH as a reference gene, and data were expressed as fold-

change (FC) compared to control DMSO samples (Livak and Schmittgen, 2001). Statistical significance was determined by the Mann Whitney test.

Statistical calculations were performed using GraphPad InStat 3.06 (GraphPad Software, Inc., San Diego, CA), and differences were considered significant at $P < 0.05$.

3. Results

3.1 AHR-target genes expression in uncultured lymphocytes from contaminated and not-contaminated cows

In uncultured bovine lymphocytes, AHR, ARNT and CYP1B1 were expressed at measurable levels, while CYP1A1 and AHRR could not be detected. In particular, as regards sampling I, AHR and ARNT were detectable in all animals, while CYP1B1 was detectable in 17/20 cows from farm A, in 16/20 cows from farm B, and in 14/20 cows from farm C. There were no statistically significant differences in the expression of either AHR or ARNT in the animals from the three different farms (Fig. 1); by contrast, the CYP1B1 expression was higher in lymphocytes collected from animals reared in the contaminated area compared to controls (Fig. 2A). However, such a difference was statistically significant ($P < 0.05$) only for farm A, which displayed the highest bulk milk TEQ value (Fig. 2B).

Taking into account the potential up-regulation of CYP1B1 observed in lymphocytes from DL-compound exposed cows, we investigated its expression in white blood cells isolated from the same animals 8 months after the first sampling, when the bulk milk TEQ values of the two contaminated farms had greatly decreased and were closely approaching (farm A), or even below (farm B) the legal threshold limits in force at that time. No detectable CYP1B1 levels were measured in 5 out of 18 samples from farm A and 6 out of 19 samples from farm B, respectively, and only lymphocytes from 10 cows for each contaminated farm could be

used for paired statistical comparison between sampling I and II. The statistical analysis of such samples showed that CYP1B1 levels in animals from the most contaminated farm (farm A) exhibited a reduced gene expression in sampling II compared to sampling I (Fig. 3). By contrast, no statistical significant differences were observed for farm B lymphocytes.

3.2 CYP1A1 and CYP1B1 induction in cultured lymphocytes from contaminated and not-contaminated cows

The above results showed a marked decrease in lymphocyte target gene expression from cows reared in farm A and fed on a decontaminated diet, leading to milk DL-compound contamination close to the legally allowed limits. Based on the approach followed by Landi et al. (2003) on human lymphocytes, we decided to investigate the inducibility of the AHR signaling pathway in cultured lymphocytes. To this aim, primary cultures of lymphocytes sampled from cows reared either in farm A or C (N = 11 each) were treated with PCB126 under the same conditions reported by Girolami et al. (2011), and assayed for the expression of CYP1A1 and CYP1B1, the two most responsive genes. Both genes were basally expressed in cultured lymphocytes from all sampled animals and up regulated by PCB126; CYP1A1 was affected to a greater extent. However, the expression of CYP1 genes in lymphocytes from cows of farm A appeared to be less inducible by the DL-PCB compared to that of control animals (Fig. 4, $P < 0.05$).

4. Discussion

In a previous paper (Girolami et al., 2011), we demonstrated the presence and inducibility of the AHR signaling pathway in cultured bovine lymphocytes. The results of the present study showed for the first time the expression of some AHR-target genes in uncultured bovine peripheral lymphocytes. In line with data reported for humans, only AHR, ARNT and

CYP1B1 were readily detectable, while CYP1A1 was expressed exclusively upon cell culturing in the presence of a mitogen (Landi et al., 2003; Toide et al., 2003; van Duursen et al., 2005). The AHR signaling pathway expression profile is slightly different in rat lymphocytes, where CYP1A1 is also detectable, though at a very low level (Saurabh et al., 2010).

One of the major goals of the present study was to investigate whether the field exposure to DL-compound contaminated roughage –as assessed by bulk milk TEQ values– could affect the expression of AHR-mediated genes in circulating lymphocytes from dairy cows. Of the three genes we found to be constitutively expressed in (uncultured) bovine lymphocytes, only CYP1B1 appeared to be significantly induced (up to 40-fold when considering median values); in addition, the extent of the induction seemed to be related to the degree of DL-compound exposure, being remarkably higher in the animals reared in the most contaminated farm. As regards CYP1A1, which is reported as the most inducible gene of the AHR signaling pathway in many tissues (Abel and Haarmann-Stemmann, 2010), its expression was not detectable (and, thus, not induced) even in the lymphocytes from the most exposed cows. However, results from our study are consistent with those obtained in investigations conducted on human peripheral lymphocytes from subjects exposed to dioxins, as assessed by their plasma TEQ values. Indeed, CYP1B1 was the only induced gene in leukocytes from workers occupationally exposed at waste incinerators (Toide et al., 2003). Likewise, in a population-based survey conducted approximately 20 years after the Seveso accident (Landi et al., 2003), CYP1B1 expression in uncultured lymphocytes was positively correlated with TEQ values, whereas CYP1A1 was not detectable either in exposed or in control subjects. To the best of our knowledge, the up regulation of CYP1A1 in lymphocytes upon stimulation of the AHR signaling pathway *in vivo* has been demonstrated only in rats, and following the administration of non DL-compounds (i.e. 3-methylcolantrene and diesel exhaust particles)

(Saurabh, 2010; Srivastava, 2012). The reason why CYP1A1 expression in uncultured lymphocytes is not affected by DL-compounds exposure in humans and cows remains to be established.

The apparent correlation between lymphocyte CYP1B1 expression and DL-compound exposure of dairy cows was further confirmed by the gene level decrease following the lowering of milk TEQ values as the result of the animal decontamination (results of sampling II, farm A). Although CYP1B1 expression could be influenced by several factors, including potential polymorphisms that could play an important role in the interindividual variability and sensitivity to inducing agents (Landi et al., 2005; van Duursen et al., 2005), such data further corroborate the hypothesis of the *in vivo* activation of the AHR signaling pathway in blood cells collected from exposed cows (farm A). The high degree of variability in gene expression observed in farm A cows, which can be appreciated in the distribution of raw data (sampling I), might be also ascribed to different physiological conditions, such as pregnancy or lactation stage, that could influence the extent of DL-compound re-distribution and excretion (Brambilla et al., 2008; Hirako et al., 2005; Hirako, 2008). Breed might also have affected the basal expression and possibly the inducibility of some of the examined AHR-mediated genes. While breed-related variation in CYP1A expression have been reported in mice (Casley et al., 1997), little is known in cattle, in which studies pertaining to CYP3A only have been conducted (Dacasto et al., 2005). In addition, very limited information is available about the influence of breed on CYP1A inducibility in farm animals. In one of the few published reports, no statistically significant differences in the rate of CYP1A-mediated 7-ethoxyresorufin O-deethylase (EROD) activity were detected in PCB 126-induced primary hepatocyte cultures from four different chicken breeds (Kennedy et al., 1996).

As regards the *in vitro* inducibility of the AHR pathway in cultured lymphocytes from contaminated cows, our results show that the previous *in vivo* exposure to DL-compounds

could modulate CYP1A1 and CYP1B1 induction in a negative way. Although, as expected, (Girolami et al., 2011), PCB126 was able to increase the expression of both genes, the response was significantly reduced by almost 30% in lymphocytes collected from the exposed animals compared to controls. The above results are in line with data reported for human cultured lymphocytes where the extent of TCDD-mediated CYP1B1 induction, as assessed by measuring EROD activity, was moderately decreased in subjects displaying higher TCDD plasma levels (Landi et al., 2003). Such a finding is difficult to explain. It might be speculated that the lower inducibility of the AHR-related target gene expression observed in lymphocytes from previously exposed individuals upon the *in vitro* treatment with DL-compounds could be due to a sort of “desensitization” of the pathway, which is already activated. This hypothesis could be in line with the reduction of AHR expression highlighted in lymphocytes from Seveso’s population (Landi et al., 2003), although such a finding has not been detected in our study. Further research is needed to unravel the mechanism(s) underlying such a phenomenon.

In conclusion, the up-regulation of lymphocyte CYP1B1 gene expression was reported for the first time in dairy cows as a result of the *in vivo* field exposure to DL-compound contaminated feedstuffs. In addition to a number of limitations discussed above, it should be recognized that neither the assessment of animal contamination was conducted on an individual basis nor the presence of other inducing pollutants, such as certain polycyclic aromatic hydrocarbons, was monitored (Guiavarc'h et al., 2011). Nevertheless, it is worth noting that contaminated cows from the present study displayed both in blood cells and in plasma a number of alterations that have been associated to DL-compound exposure in farm or laboratory species, such as chromosomal fragility and increased oxidative stress (Genuardo et al., 2012; Perucatti et al., 2006; Slezak et al., 2000; Son et al., 2003; Spagnuolo et al., 2011). In particular, chromosomal abnormalities in leukocytes, impairment of the plasma

antioxidant defense system, and changes in low molecular weight serum protein profile have been the subject of separate reports (Bertarelli et al., 2010; Di Meo et al., 2011; Spagnuolo et al., 2012). Further experimental research is warranted investigating the correlation between the observed changes and the individual level of contamination-decontamination (i.e. through single milk or plasma TEQ values) before being able to conclude that AHR-target genes in circulating lymphocytes could be utilized for biomonitoring purposes in cattle.

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Table 1

Dioxin and DL-PCB levels in bulk milk from dairy cows reared in different areas of Susa Valley (northern Italy) at two time-points.

	Sampling I			Sampling II		
	A	B	C	A	B	C
WHO-PCDD/PCDF-TEQ (pg/g of fat)	1.66	1.11	1.2	0.53	0.69	–
WHO-PCB-TEQ (pg/g of fat)	16.09	7.45	0.55	8.12	4.15	–
WHO-PCDD/PCDF-PCB-TEQ (pg/g of fat)	17.75	8.56	1.75	8.65	4.84	–

WHO: World Health Organization; TEQ: toxic equivalents; PCDD: polychlorinated dibenzo-para-dioxins; PCB: polychlorinated biphenyls; PCDF: polychlorinated dibenzo-furans.

Allowed levels of WHO-PCDD/F-TEQ and WHO-PCDD/F-PCB-TEQ in force at that time were 3.0 and 6.0 pg/g of fat, respectively.

Figures

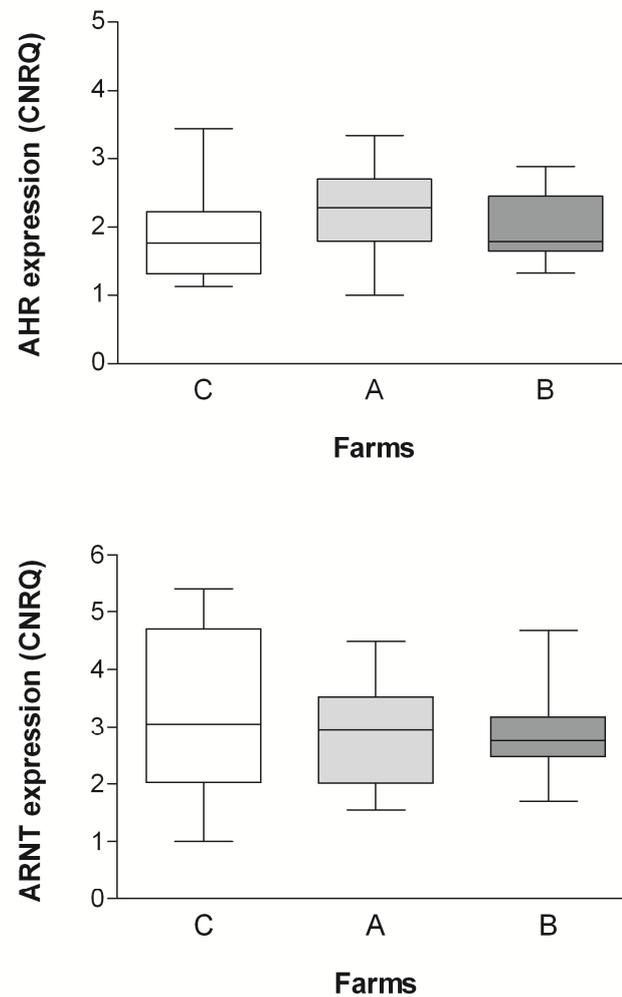


Fig. 1. Box and Whiskers plots showing the relative gene expression of AHR and ARNT in uncultured lymphocytes from dairy cows exposed (A and B) or not exposed (C) to dioxins and DL-PCBs (n = 20).

CNRQ: calibrated normalized relative quantity.

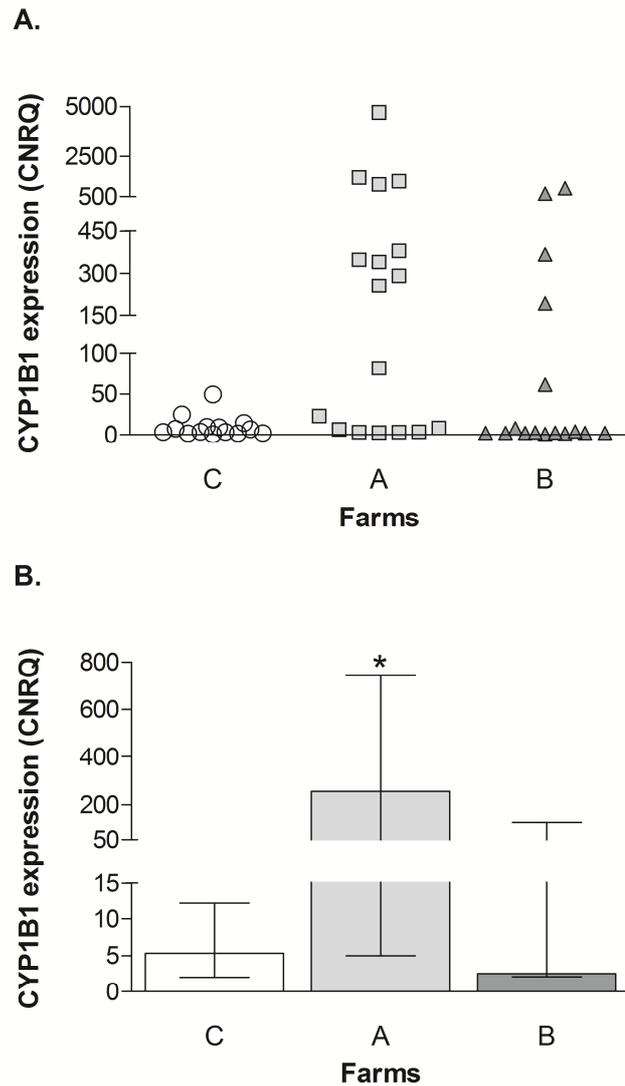


Fig. 2. Relative gene expression of CYP1B1 in uncultured lymphocytes from dairy cows exposed (A and B) or not exposed (C) to dioxins and DL-PCBs (sampling I), depicted as distribution of raw values (panel A), and medians with interquartile ranges (panel B). Asterisk denotes a significant difference ($p < 0.05$) using Kruskal–Wallis ANOVA test followed by Dunn's multiple comparison test (group A, $n = 17$; group B, $n = 16$; group C, $n = 14$). CNRQ: calibrated normalized relative quantity.

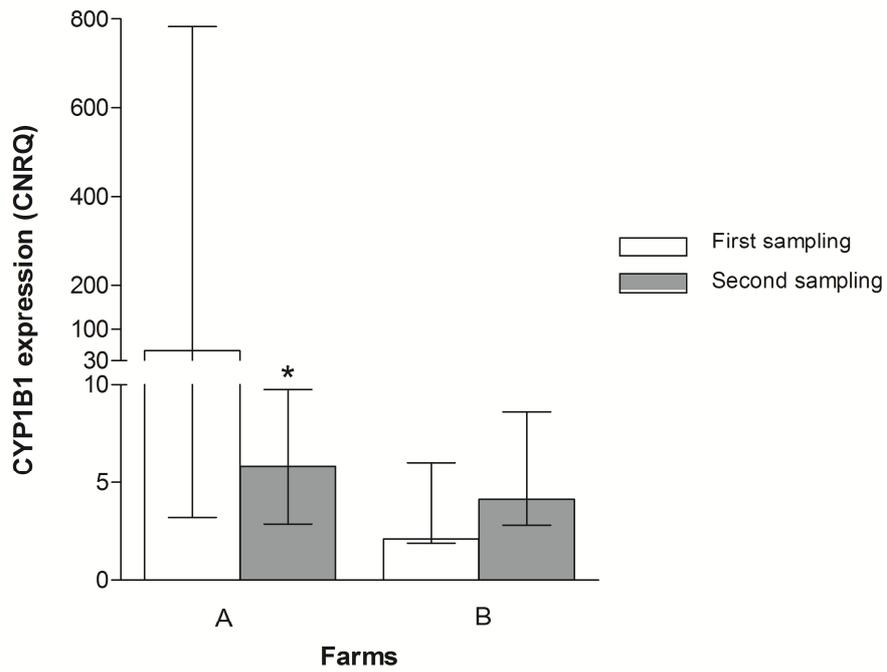


Fig. 3. Relative gene expression of CYP1B1 in uncultured lymphocytes from dioxin and DL-PCB exposed dairy cows (farms A and B; sampling I and II); data are depicted as medians with interquartile ranges. Asterisk denotes a significant difference ($p < 0.05$) using Wilcoxon Signed-Rank Test. $N = 10$ for each farm.

CNRQ: calibrated normalized relative quantity.

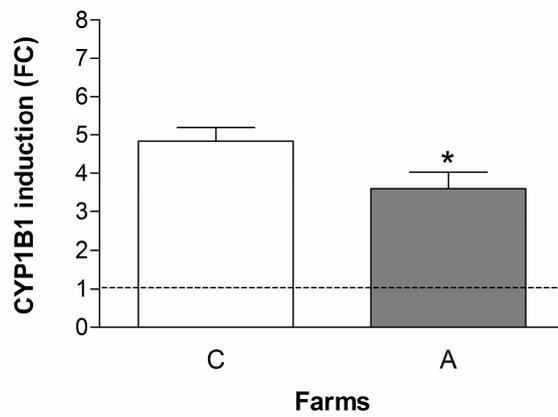
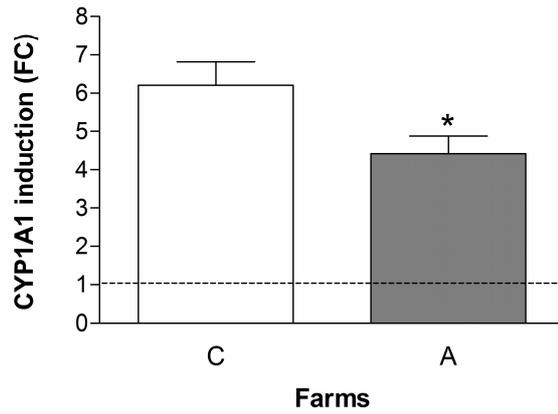


Fig. 4. CYP1A1 and CYP1B1 induction in cultured blood lymphocytes from dairy cows of farms A and C, following treatment with PCB126 (100nM). Fold change (FC) levels are expressed as mean \pm SEM. The dashed line indicates the level of induction of control cells treated with DMSO. Asterisk denotes a significant difference ($p < 0.05$) from controls (group C) using Mann Whitney test ($n = 11$).