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The aryl hydrocarbon receptor agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin alters the circadian rhythms, quiescence, and expression of clock genes in murine hematopoietic stem and progenitor cells.

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Running Title: Ah Receptor and Circadian Rhythms in Hematopoiesis

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Abbreviations Used: AhR, aryl hydrocarbon receptor; AhRR, aryl hydrocarbon receptor repressor; Arnt, aryl hydrocarbon receptor nuclear translocator; CFU-GEMM, colony-forming unit granulocyte erythrocyte macrophage megakaryocyte; CFU-GM, colony-forming unit granulocyte macrophage; CMP, common myeloid progenitors; GMP, granulocyte macrophage progenitors; HSC, hematopoietic stem cells; HPC, hematopoietic progenitor cells; HPP-CFC, high proliferative potential colony-forming cells; LSK, $Lin^{-}Sca\text{-}1^{+}cKit^{+}$; MEP, megakaryocyte erythrocyte progenitors; SCF, stem cell factor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ZT, Zeitgeber times

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), an aryl hydrocarbon receptor (AhR) agonist, has been identified as a potent immuno-hematopoietic toxicant with the ability to alter the number of Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) bone marrow cells, a population enriched for murine hematopoietic stem cells. The biology of these cells is governed by circadian rhythms and TCDD has been shown to disrupt circadian rhythms of other biological endpoints. We investigated the effect of TCDD on the circadian rhythms of hematopoietic precursors. Female C57Bl/6 mice were treated with a single oral dose of 10μg/kg TCDD. Five days later, bone marrow was harvested every four hours for twenty-four hours and stained for specific hematopoietic populations using fluorescently-labeled antibodies. In addition, cells were placed into semi-solid culture to measure different functionally-defined populations. Activation of the AhR by TCDD elicited disruptions in the rhythms of LSK cell numbers and phenotypically-defined myeloid and erythroid precursors. Simultaneous DNA and RNA staining revealed an abnormal *in vivo* rhythm of $\%G_0$ LSK cells, suggesting disruption of stem cell quiescence. Finally, quantitative RT-PCR revealed that expression of AhR and Arnt mRNA within enriched hematopoietic precursors oscillates with a circadian period. Modest changes in the twenty-four hour expression of mPer1 and mPer2 mRNA and increased AhR repressor mRNA following TCDD exposure suggest a direct effect on the molecular machinery responsible for these rhythms. Together, these data demonstrate that activation of the AhR by TCDD disrupts the circadian rhythms associated with murine hematopoietic precursors.

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The aryl hydrocarbon receptor (AhR) is a bHLH protein and member of a relatively homologous, but functionally diverse, family of proteins containing one or more PAS (Per-Arnt-Sim) domains. This family contains the AhR's heterodimerization partner the aryl hydrocarbon receptor nuclear translocator (Arnt) as well as hypoxia inducible factor-1α (HIF-1α) and principal components of circadian rhythm regulators, Clock and BMAL1. In addition to being a highly-conserved, developmentally-regulated, and ligand-activated transcription factor (Hahn, 2002; Williamson *et al*., 2005), the AhR is also the protein mediator for the action of several organic toxicants, typified by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Altered immuno-hematopoietic parameters, including both humoral and cellular, are among the most consistently observed toxicities following activation of the AhR by xenobiotic agonists such as TCDD. A hallmark of AhR-mediated toxicity is a persistent atrophy/aplasia of the thymus. Although this is may be partly due to effects on thymocyte proliferation (Laiosa *et al*., 2003), other data suggest that other upstream cellular targets exist that may contribute to this effect. In particular, the sensitivity of Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) bone marrow cells, a population highly enriched for multipotent hematopoietic stem cells (HSC), to TCDD was demonstrated (Murante and Gasiewicz, 2000; Sakai *et al*., 2003; Staples *et al*., 1998). This is the most immature population of hematopoietic cells observed to be altered by TCDD treatment. Although predominantly quiescent, HSC are characterized by a robust proliferative capacity and the ability for self-renewal. In addition, these cells are defined by the ability to differentiate into all of the blood lineages by first committing to hematopoietic progenitor cells (HPC) that are themselves committed to either the

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lymphoid or myeloid lineages, common lymphoid progenitors (CLP) or common myeloid progenitors (CMP), respectively. While CLP further differentiate into B- and T-cells or NK-cells (Kondo *et al*., 1997), CMP differentiate to form granulocyte macrophage progenitors (GMP) and megakaryocyte erythrocyte progenitors (MEP) (Akashi *et al*., 2000).

A single dose of TCDD has been shown to increase the number of marrow LSK cells, although their ability to reconstitute an irradiated mouse is impaired (Murante and Gasiewicz, 2000; Sakai *et al*., 2003). Furthermore, the use of irradiation chimeras demonstrated that the presence of the AhR in the hematopoietic cells, and not supportive stroma, was essential for both the TCDD-elicited increase in LSK cells and their functional impairment (Staples *et al*., 1998; Sakai *et al*., 2003). This is particularly important because of the tightly regulated and critical interactions between hematopoietic precursors and the non-hematopoietic stroma. These findings along with measurable levels of AhR mRNA and protein in these cells (van Grevenynghe *et al*., 2005) indicate that the AhR may have some normal function in HSC. In addition, human HSCs are AhR-dependent targets for carcinogenic polycyclic aromatic hydrocarbons (van Grevenynghe *et al*., 2005).

Many members of the PAS family, including the two murine Period homologues mPer1 and mPer2, are involved in controlling circadian rhythms (Zheng *et al*., 2001). These rhythms occur with an approximate twenty-four hour period and are a temporal compartmentalization that governs much of vertebrate physiology. All aspects of

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hematopoiesis, including proliferation and differentiation, are controlled by circadian rhythms. For example, the number of human $CD34^+$ bone marrow cells as well as those in S-phase has been shown to be rhythmic (Abrahamsen *et al*., 1998). Rhythmicity in numbers of erythroid progenitors, BFU-E and CFU-E, and myeloid progenitors, CFU-GEMM and CFU-GM, have been identified (Aardal and Laerum, 1983; Smaaland *et al*., 1992; Stevold *et al*., 1988; Wood *et al*., 1998). Although it is likely that at least part of their rhythmicity is regulated by circadian-controlled circulating factors, these cells express all of the machinery required for an intrinsic circadian clock and maintain rhythmicity *in vitro* (Bourin *et al*., 2002; Tsinkalovsky *et al*., 2005).

The AhR itself has been linked to circadian physiology. Both mRNA and protein levels of AhR in rat liver oscillates in a circadian manner (Huang *et al*., 2002; Richardson *et al*., 1998). Not surprising, the level of *CYP1A1* mRNA, one of the AhR-regulated genes, has a rhythm that is anti-phase to the AhR. A single acute exposure to TCDD also disrupted circadian-controlled rest/activity rhythms in mice, as well as the rhythms associated with the expression of circadian genes *Per1* and *BMAL1* in the hypothalamus (Miller, 1999). The purpose of these experiments was to test the hypothesis that dysregulation of the AhR signaling pathway resulting from exposure to a persistent AhR agonist TCDD could disrupt the circadian rhythms of antigenically- and functionally-defined hematopoietic precursors.

Materials and Methods

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Animals. Five-week old female C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for a period of one week. All animals were cared for under a protocol approved by the University Committee on Animal Resources. They were provided food and water *ad libitum*. Lights were maintained on a twelve-on/twelve-off schedule, turning on and off at 6:00 am and 6:00 pm. These times correspond with Zeitgeber times (ZT) 0 and 12, respectively.

TCDD Exposure. Mice were gavaged with 10μg/kg TCDD in 5μL olive oil per 1g mouse weight, or oil alone five days prior to the first timepoint. In all studies, time points consisted of ZT6, 10, 14, 18, 22, and 2. The five day time point was predetermined to be optimal in generating an effect on the percent of quiescent Lin^- $Sca\text{-}1^+$ cells in the BM; this effect was maximal between days 3 through 7 following exposure (not shown).

Bone Marrow Cell Isolation. Bone marrow cells were flushed from both femurs and tibiae with PBS + 2% FBS + 1% penicillin/streptomycin as previously described (Wyman *et al.,* 2002; Laiosa *et al*., 2003). The bone marrow cells were placed in to a single cell suspension before passing through an 80-μm nylon mesh (TETKO Inc., Briarcliff Manor, NY). Cell pellets were treated with erythrocyte lysis buffer (0.17 M NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA) and the remaining cells were washed and counted.

Immunophenotyping. One million BM cells from vehicle- and TCDD-treated mice were blocked with anti-FcR antibody in stain buffer (PBS + 1% FBS + 0.1% NaN_3) for 20 minutes on ice and then incubated for 30 minutes with predetermined saturating

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concentrations of the following fluorescently-conjugated antibodies: FITC-conjugated anti-CD34, Thy-1, Sca-1, Gr-1, and cKit; PE-conjugated anti-Sca-1 and FcR; APC-conjugated anti-c-Kit; Biotin-conjugated anti-Mac-1, Gr-1, CD8a, CD3ε, Ter119, B220, Sca-1, and IL-7R (BD Biosciences, San Jose, CA). Specific cell populations were detected using the following surface phenotypes as previously published by others (Akashi *et al*., 2000; Kondo *et al*., 1997): HSC (Lin^- $Thy\text{-}1^+$ $Sca\text{-}1^{hi}$ $c\text{-}Kit^+$), CMP (Lin^- $CD34^+$ FcR^{lo} $IL7R^-$ $Sca\text{-}1^-$ $c\text{-}Kit^+$), GMP (Lin^- $CD34^+$ FcR^{hi} $IL7R^-$ $Sca\text{-}1^-$ $c\text{-}Kit^+$), and MEP (Lin^- $CD34^-$ FcR^{lo} $IL7R^-$ $Sca\text{-}1^-$ $c\text{-}Kit^+$). In all cases the lineage cocktail included antibodies to Mac-1, Gr-1, CD8a, CD3ε, Ter119, and B220. Streptavidin-PerCP was used a secondary fluorescent agent. Cells were rinsed with staining buffer and fixed with 0.1% paraformaldehyde prior to running on the flow cytometer. As compared to our previous studies (Murante and Gasiewicz, 2000; Staples *et al*., 1998), the use of the Thy-1 marker allows further definition of the Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) population to include those cells that are phenotypically- and functionally-defined as long-term (LT) and short-term (ST) HSC. Cells were run (at least 50,000 events per sample) on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with CellQuest software (BD Biosciences). Gates were set similarly as previously described (Staples *et al*., 1998; Murante and Gasiewicz, 2000; Akashi et al., 2000; Kondo et al., 1997) and data were taken as percentages of cells in the viable gates.

Cell Isolation for RNA Analysis. Total BM was harvested from both legs (femur + tibia) and blocked with anti-FcR in stain buffer. The cells were then stained with biotinylated anti-lineage (Mac-1, Gr-1, B220, CD8α, CD3ε, Ter119) followed by

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streptavidin-microbeads (Miltenyi Biotec; Auburn, CA). Lineage depletion was performed using an AutoMACS magnetic cell sorter (Miltenyi Biotec). For certain experiments, $Lin^-Sca\text{-}1^+$ and $Lin^-Sca\text{-}1^-$ cells were collected by staining the Lin^- fraction with anti-Sca-1 microbeads (Miltenyi Biotec). Collection of $Sca\text{-}1^+$ and $Sca\text{-}1^-$ cells were performed using the sensitive positive sort function of the AutoMACS. Purity of fractions was assessed using flow cytometric analysis and determined to be > 95%.

Colony-Forming Semi-Solid Assays. To measure high proliferative potential colony-forming cells (HPP-CFC), twenty thousand BM cells were suspended in 1mL of IMDM supplemented with 1% methylcellulose, 25% FBS, 1% BSA, 100μM 2-mercaptoethanol, and 2mM L-glutamine (Stem Cell Technologies, Vancouver, BC, Canada). To the media was added 250ng/mL CSF-1, 50ng/mL IL-3, 50ng/mL IL-1α, and 50ng/mL SCF (R&D System, Minneapolis, MN). CFU-GEMM and CFU-GM were measured using methylcellulose based media containing SCF, IL-3, IL-6, and erythropoietin or SCF, IL-3, and IL-6, respectively (Stem Cell Technologies). All plates were incubated at 37°C in 5% CO_2 and scored fourteen days later. HPP-CFC were scored by the presence of macroscopic colonies (>0.5mm in diameter). CFU-GEMM and CFU-GM were scored as colonies composed of granulocytes, erythrocytes, and monocytes or granulocytes and monocytes, respectively.

Cell Cycle Analysis. One million Lin^- BM cells were stained with FITC-conjugated anti-cKit and AF647-conjugated anti-Sca-1. Afterwards, cells were stained with 7-aminoactinomycin D (7AAD) (Molecular Probes) at room temperature for twenty

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minutes followed by Pyronin Y (PY) on ice in nucleic acid staining solution (0.1M phosphate-citrate buffer + 0.15M NaCl + 5mM sodium EDTA + 0.5% BSA + 0.02% saponin) for 10 minutes. Cells were analyzed using a FACSCalibur flow cytometer and data analyses were performed using CellQuest software.

Quantitative RT-PCR. Total RNA was isolated from Lin^- $Sca\text{-}1^+$ and Lin^- $Sca\text{-}1^-$ BM cells at the indicated time points five days following *in vivo* exposure to TCDD using the RNEasy Mini kit (Qiagen, Valencia, CA) as instructed. RNA amplifications were performed using WTA (NuGEN Technologies, San Carlos, CA). cDNA was created using the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA) as instructed and PCR was performed using a 7900 HT (Applied Biosystems, Foster City, CA). TaqMan gene expression assays (Applied Biosystems) were used to measure the following targets: AhR, Arnt, AhRR, mPer1, mPer2, p21, p27, and GAPDH. Data analyses were performed using the comparative Ct method.

Statistical Analysis. A two-way ANOVA (Excel) was performed on each set of 24 h data to determine treatment-dependent differences. When the ANOVA indicated differences between treatment groups, the Students t-test was used to determine differences between control and experimental groups at designated time points. Values of $p \leq 0.05$ were considered statistically significant.

Results

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TCDD disrupts the circadian rhythm of hematopoietic precursor numbers. The numbers of HSC and HPC have been established to fluctuate with a circadian period (Abrahamsen, 1998). With this in mind and since prolonged activation of the AhR by TCDD increases the number of murine LSK BM cells (Murante and Gasiewicz, 2000; Sakai *et al*., 2003), we measured the effect of AhR activation on the circadian rhythms associated with these cells. Five-week old female C57Bl/6 mice received by gavage a single 10μg/kg dose of TCDD. Five days later, bone marrow was collected every four hours for twenty-four hours and stained with fluorescently-labeled antibodies. As indicated in Figure 1, Thy-1^{lo} LSK cells, a population that contains all of the murine HSC, are characterized by a near 3-fold difference between the minimum value and the maximum value in vehicle-treated mice. Following treatment with TCDD, there is an overall increase in the number of Thy-1^{lo} LSK cells (Fig. 1) throughout the 24-hour period (ANOVA p value < 0.001). There were no statistical differences observed in total bone marrow cellularity at any time point (not shown). A comparable TCDD-induced increase in this population of progenitor cells was observed in similar experiments carried out over the course of about one year. These studies were carried out due to known seasonal variations in these diurnal patterns. Occasionally, these experiments conducted at different seasons also suggested abnormal period and/or phase shifts following exposure toTCDD (data not shown).

Since there is a considerable body of literature demonstrating circadian rhythmicity in myeloid and erythroid precursors (Aardal and Laerum, 1983; Smaaland *et al*., 1992; Stevold *et al*., 1988; Wood *et al*., 1998), these studies were extended to examine effects

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on these populations. CMP, GMP, and MEP all were characterized by apparent rhythms in control animals (Fig. 2). Five days following TCDD exposure, the rhythms of all three populations were altered (ANOVA p value < 0.001), demonstrating changes in phase and magnitude that are both time-of-day- and cell-type specific (Fig. 2). In addition to measuring antigenically-defined hematopoietic precursors, we performed methylcellulose based *ex vivo* assays to determine effects on functional capacity by measuring high proliferative potential colony-forming cells (HPP-CFC), colony-forming unit granulocyte erythrocyte macrophage megakaryocyte (CFU-GEMM) and CFU-granulocyte macrophage (CFU-GM). As was seen with those antigenically-defined cells, these functionally-defined populations were all characterized by diurnal rhythms (Fig. 3). Although time-of-day-specific changes may have occurred following TCDD treatment, there were no overall significant treatment-dependent differences when the 24-hour data sets for these colony assays were compared. Together, these data demonstrate that a single oral dose of TCDD is sufficient to cause a modification in the circadian rhythms of murine hematopoietic precursors, as well as overall changes in their numbers. These changes can be both positive and negative, with time points during a daily cycle that are absent of differences between treatment groups.

TCDD disrupts the circadian rhythm of LSK quiescence. Simultaneous staining for RNA and DNA allows discrimination between G_0 and G_1 phase cells. This is an important distinction to make when investigating HSC because of their relatively high representation in quiescence (G_0) under normal homeostatic conditions. In addition, G_0 human $CD34^+$ cells, a population enriched for human HSC, were found to be more

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highly enriched for long-term culture initiating capacity, indicating functional differences between cells differing in their cell cycle status (Gothot *et al*., 1997). As indicated in Figure 4, the number of G_0 LSK cells in control mice exhibits a pattern suggesting a bi-phasic effect, with two periods of diminished quiescence occurring at the beginning of both perceived day and perceived night. Following TCDD treatment, the daily cycle of quiescent cells was disrupted (ANOVA p value < 0.001). The most obvious difference is at the period of late perceived night when the greatest percentage of the control LSK cells are in quiescence. Here more LSK cells in TCDD-treated mice exit quiescence at ZT22 such that the ratio of quiescent cells to actively cycling cells is at its lowest of anywhere on the two curves. Comparing the data specifically between ZT18 and ZT2 also suggests the possibility that the period of the rhythm has been compressed. Together , these data suggest that one possible mechanism by which TCDD-induced AhR activation alters the numbers and rhythms of primitive hematopoietic progenitors is the disruption of their movement in and out of quiescence. These data are also consistent with other studies from our lab showing increased BrdU incorporation into LSK marrow cells following TCDD treatment (manuscript submitted).

TCDD disrupts the circadian rhythm of Lin$^-$ Sca-1$^+$ gene expression. Circadian rhythms are controlled by tightly regulated molecular clocks, at the heart of which are several PAS transcription factors, including the murine Period homologues mPer1 and mPer 2, that are structurally similar to the AhR and its dimerization partner Arnt (Zheng *et al*., 2001). These proteins act as important positive regulators of the circadian clock. Very recently, a second putative AhR DNA response element has been identified

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upstream of several genes including mPer2 (Boutros *et al*., 2004). We therefore sought to determine whether exposure to TCDD could disrupt the expression of these genes in Lin^- $Sca\text{-}1^+$ bone marrow cells. The data presented in Figure 5 agree with previous studies demonstrating that mPer1 and mPer2 are expressed in murine marrow Lin^- $Sca\text{-}1^+$ cells and that this expression is itself circadian in manner (Chen *et al*., 2000; Tsinkalovsky *et al*., 2005). Both mPer1 and mPer2 are characterized by rhythms that have maximal expression near ZT14 and ZT23, respectively. These patterns were disrupted by TCDD treatment (ANOVA p value < 0.05) with peaks being truncated by nearly 50%. The cyclin dependent kinase inhibitors p21 and p27 also have been demonstrated to be both TCDD responsive and regulated with circadian rhythmicity (Bjarnason *et al*., 1999; Cheng *et al*., 2000; Enan *et al*., 1998; Kolluri *et al*., 1999). However, analysis of their mRNA expression over twenty-four hours revealed only modest rhythmicity in Lin^- $Sca\text{-}1^+$ cells and no changes with TCDD exposure (data not shown). Together, these data support previous findings that hematopoietic precursors express circadian related genes. These data also indicate that this expression is susceptible to dysregulation by TCDD.

Finally, it has been demonstrated by others that both AhR mRNA and protein expression are circadian regulated in several other tissues including rat liver (Huang *et al*., 2002; Richardson *et al*., 1998). We sought to determine whether the AhR and Arnt are expressed with circadian rhythmicity in immature hematopoietic cells. Total RNA was isolated from Lin^- $Sca\text{-}1^+$ cells collected over a twenty-four hour period and qRT-PCR was used to measure AhR and Arnt mRNA. Both have maximal expression

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occurring during subjective mid-day (near ZT10) with minimal expression occurring during the hours surrounding the transition from dark to light (Fig. 6). These curves were not significantly modified following TCDD exposure (data not shown). The AhR repressor (AhRR) is a recently characterized TCDD-inducible member of the AhR signaling pathway that competes with the AhR for binding to both AhRE and Arnt and acts as a negative regulator of AhR-elicited transcriptional activity (Baba *et al*., 2001). TCDD exposure up-regulated AhRR in murine Lin^- $Sca\text{-}1^+$ BM cells 20-fold in a time-of-day dependent manner. In addition, although the pattern of AhRR mRNA expression is characterized by a single, but very modest, maximal time of expression near ZT10 in control animals, TCDD-treated animals have an additional peak of high expression occurring approximately twelve hours later (Fig. 7). Together, these data demonstrate that the members of the AhR signaling pathway are expressed in a circadian manner in murine hematopoietic precursors and that the expression of the AhRR is susceptible to TCDD-induced dysregulation.

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Discussion

Processes regulating the numbers of hematopoietic stem and progenitor cells are under the control of circadian timing mechanisms (Aardal and Laerum, 1983; Abrahamsen *et al*., 1998; Smaaland *et al*., 1992; Stevold *et al*., 1988; Wood *et al*., 1998). As such, the number of murine hematopoietic progenitors can change as much as 2- to 3-fold during the course of a single day. Although these rhythms demonstrate a change in a specific antigenically- or functionally-defined cell type, it is important to understand that this likely does not represent changes in the number of actual cells. Instead, these daily cycles likely represent changes in the expression of HSC- and HPC-specific proteins, as well as the functional potential of those cells, two events that are not mutually exclusive. For example, rhythmic expression of cKit, the receptor for SCF, would be observed as oscillating numbers of Lin^- $Sca\text{-}1^+$ $cKit^+$ cells. This daily plasticity for the complement of proteins expressed on the surface of HSC and HPC would in turn create a time-dependent *in vivo* functional potential. This would also affect their ability to respond to *in vitro* assay conditions that include SCF, creating measurable rhythms in the numbers of SCF-dependent colony forming cells. Diurnal rhythms have been demonstrated for other growth factor receptors, such as the surface expression of interferon-α/β receptor (IFNαR) on hepatocytes, lymphocytes, and implanted melanoma cells (Takane *et al*., 2001; Takane *et al*., 2002). The maximal expression of IFNαR on tumor cells corresponded with an increase in the percent of those cells in S-phase, indicating a relationship between cell cycle transit and specific growth factor signaling (Takane *et al*., 2001). The circulating levels of soluble receptors for TNF, IL-6, and IL-2 are all rhythmic (Haack *et al*., 2004). The responsiveness of murine myeloid progenitors to IL-

3, GM-CSF, and G-CSF was also found to be circadian-dependent (Perpoint *et al*., 1995).

We have presented data that for the first time indicate circadian rhythms in the numbers of specific antigenically-defined populations of murine hematopoietic progenitor cells. Thy-1^{lo} LSK cells, a population of cells enriched for murine HSC, as well as three populations of Lin^{-} cKit^{+} myeloid precursors CMP, GMP, and MEP, all appeared to have diurnal rhythms in vehicle-treated C57Bl/6 mice (Figs. 1 and 2). Data for MEP and GMP suggest rhythms that were anti-phase to each other, supporting data that differentiation into the different lineages is separated in time (Wood *et al*., 1998). Hypothetically, a measurable plasticity may exist between erythroid and myeloid progenitors, with a single cell changing surface levels of proteins such as FcR and CD34 so that it meets the criteria for both GMP and MEP at different times of the day. The anti-phase nature of the curves for those two populations supports this notion (Fig. 2). This kind of temporal compartmentalization provides for unique periods of specific timing and duration when cellular and extracellular factors required for a specific differentiation program are likely up-regulated. Properly timed circadian rhythms are hypothesized to be energetically advantageous to the organism, allowing production of signaling molecules and receptors only when they are required. Five days after TCDD exposure there appeared to be a shift in the timing of the GMP and MEP rhythms; although those two specific curves remained anti-phase to one another, adding support to our hypothesis that these are indeed the same cells. Although the absolute minimum numbers of these two populations do not change following TCDD treatment, the

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maximal values increase roughly 2-fold (Fig. 2). This increase in the number of myeloid-erythroid progenitors does not necessarily precede increased hematopoietic potential. It is likely that in control bone marrow, the expression of progenitor-specific markers is timed to correspond with extracellular signals required for further differentiation. TCDD may decouple these two sets of events, and depending on whether those signals are positive or negative regulators, the increased numbers of GMP and MEP can be characterized by more, less, or unchanged function. It is therefore critical that future experiments investigate the direct consequences of shifted circadian rhythms to the outcome of hematopoietic progenitors, i.e. their ability to differentiate into more committed lineage cells. In this regard, it is noteworthy that other data suggest a TCDD-elicited shift toward the granulocyte/macrophage lineage at the expense of lymphoid and erythroid lineages in murine bone marrow (manuscript submitted). This is consistent with published data showing a decrease in the production of immature B and T cells in marrow following TCDD exposure (Thurmond *et al*., 2000; Fine *et al*., 1990), but increased numbers and proliferation of $Lin^-/Sca\text{-}1^+/cKit^+$ stem/progenitor cells as indicated in this manuscript. At present it is not known if or how modulated hematopoietic rhythms may be related to these outcomes.

In addition to numbers of specific cells, the apparent function and proliferation of HSC and HPC is also temporally dependent. The cell cycle of both primitive hematopoietic progenitors and other cell types progresses with a twenty-four hour period and is linked to the co-expression of circadian associated genes. Notably, the ability of transplanted murine bone marrow to engraft is influenced by circadian time (D'Hondt *et al*., 2004).

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Thus, cell cycling and functional potential are likely linked. Consistent with this, investigators have shown that HSC isolated while in the active phases of the cell cycle are less able to home to bone marrow and spleen (Jetmore *et al*., 2002). It is therefore not surprising that the rhythm of S-phase HSC and HSC engraftment are anti-phase to each other (D'Hondt *et al*., 2004) and that the number of colony forming unit-spleen (CFU-S) are measured with a circadian period (Stevold and Laerum, 1988). We have shown a TCDD-elicited disruption in the quiescence of LSK cells (Fig. 4). These data are important for several reasons. First, the regulation of HSC division is tightly regulated with the majority (70-85%) of cells quiescent under normal 'resting' conditions; a phenomenon that is critical for the maintenance of hematopoietic potential throughout the lifespan of the organism. Second, this alteration in the functional composition of the bone marrow may explain reported data indicating that TCDD, although increasing their number, diminished the ability of murine LSK/CD34$^-$ cells (functionally defined as long-term reconstituting HSCs) to reconstitute an irradiated recipient mouse (Sakai *et al*., 2003). Again, this result may not be surprising if TCDD treatment increases the cycling of HSCs and actively cycling cells are less able to home to bone marrow (Jetmore *et al*., 2002).

The proteins mPer1 and mPer2 are important positive arms of the circadian clock. Targeted mutation of their genes results in abnormal circadian rhythms (Zheng *et al*., 2001). However, because the expression of *mPer2* normally increases and decreases daily, measuring the effect of TCDD at just one time point can lead to a misinterpretation of the data. When *mPer1* and *BMAL1* mRNA expression were

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monitored in the hypothalamus from TCDD-treated mice at multiple time points over 24 hours, it was found that the overall rhythm was phase-shifted (Miller *et al*., 1999). Rather than a change in the phase of their rhythms, we observed changes in the magnitude of both *mPer1* and *mPer2* mRNA in Lin^- $Sca\text{-}1^+$ BM cells exposed *in vivo* to TCDD (Fig. 5). Specifically, their respective maximal expression were reduced by roughly 50%. Although it is unclear what effect this reduction would have on these cells, it is plausible that this transient modulation in *mPer* mRNA is significant enough to disrupt normal cellular function. In addition, although TCDD has been identified as a tumor promoter, there is currently no data providing a convincing mechanism by which this happens. Notably, *mPer* mutant mice are more susceptible to γ-irradiation induced tumorogenesis (Fu *et al*., 2002). The incidence of lymphoma and tumors in several tissues was nearly 15-fold higher in *mPer2* mutant animals, leading to early mortality. Additionally, a chromosomal translocation fusing ETV6 with the *hPer1* gene is associated with acute myeloid leukemia (Penas *et al*., 2003). Notably, TCDD exposure to humans has been associated with increased incidence of lymphoma and leukemia (IARC, 1997). It would be important to further investigate the specific effects of AhR-mediated dysregulation of *mPer* on murine hematopoietic progenitors.

Although we and others have referred to the action of TCDD on the AhR as activation, evidence suggests this may not always be functionally the case. When many types of cells are exposed to TCDD, both *in vitro* and *in vivo,* there is a rapid and sustained proteosome-mediated degradation of AhR protein (reviewed in Pollenz, 2002). In many cases, a single dose of TCDD is sufficient to reduce AhR protein *in vivo* to 10-30% of

control animals in multiple tissues for up to fourteen days (Pollenz *et al*., 1998). However, despite this decrease in the level of AhR, CYP1A1 and/or CYP1B1 increase with TCDD exposure, indicating some continued AhR function or the presence of 'spare' AhR protein. Thus, TCDD may cause a rapid and sustained reduction in AhR protein while directing the remaining active AhR to particular TCDD-inducible genes, including the AhRR (Baba et al., 2001), adding an additional level of negative regulation. The possible nullification of endogenous AhR activity in HSC/HPC by TCDD is important when considering the following points. First, a putative second AhRE has been identified upstream of the mPer2 gene (Boutros *et al*., 2004). Second, we observed a time-of-day-dependent elevated number of LSK cells in AhR-null allele mice (unpublished observations) of a magnitude similar to the increase following TCDD exposure. Third, we observed that TCDD treatment substantially induces AhRR in Lin^- $Sca\text{-}1^+$ marrow cells (Fig 7). Thus, a plausible and testable explanation for some of the present data is that TCDD is inducing, either through the ability to elicit down-regulation of AhR protein and/or increased AhRR expression, an AhR-null-like state for signaling pathways regulating HSC/HPC function. Future experiments will investigate the mechanisms by which TCDD may affect distinct AhR functions in murine LSK cells.

In summary, this paper describes a novel effect of AhR activation on immature murine hematopoietic progenitors. Significant changes in the circadian rhythms associated with both the number of antigenically- and functionally-defined cell populations as well as their movement into and out of quiescence were detected. These disruptions include changes in phase, period, and magnitude, and may be related in part to altered

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expression of mPer1 and mPer2. Experiments are planned to delineate those mechanisms responsible for these changes, including establishing the relationship between mPer expression and signaling through the AhR. In addition, experiments will be designed to gain an understanding of the consequences of disrupted circadian rhythms on both normal and leukemic hematopoiesis. Finally, these data emphasize the need to evaluate daily cycles when determining effects of AhR agonists, therapeutic agents or other chemicals, on bone marrow functions.

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References

Aardal N, and Laerum O (1983) Circadian variations in mouse bone marrow. *Exp Hematol* **9**:792-801.

Abrahamsen JF, Smaaland R, Sothern RB, and Laerum OD (1998) Variation in cell yield and proliferative activity of positive selected human CD34+ bone marrow cells along the circadian time scale. *Eur J Haematol* **60**:7-15.

Akashi K, Traver D, Miyamoto T, and Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**:193-197.

Baba T, Mimura J, Gradin K, Kuroiwa A, Watanabe T, Matsuda Y, Inazawa J, Sogawa K, and Fujii-Kuriyama Y (2001) Structure and expression of the Ah receptor repressor gene. *J Biol Chem* **276**: 33101-33110.

Bjarnason GA, Jordon RC, and Sothern RB (1999) Circadian variation in the expression of cell-cycle proteins in human oral epithelium. *Am J Pathol* **154**:613-622.

Bourin P, Ledain AF, Beau J, Mille D, and Lévi F (2002) In-vitro circadian rhythm of murine bone marrow progenitor production. *Chronobiol Int* **19**:57-67.

Boutros PC, Moffat ID, Franc MA, Tijet N, Tuomisto J, Pohjanvirta R, and Okey AB (2004) Dioxin-responsive AhRE-II gene battery: identification by phylogenetic footprinting. *Biochem Biophys Res Commun* **321**:707-715.

Chen YG, Mantalaris A, Bourne P, Keng P, and Wu JH (2000) Expression of mPer1 and mPer2, two mammalian clock genes, in murine bone marrow. *Biochem Biophys Res Commun* **276**:724-728.

Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, and Scadden DT (2000b) Hematopoietic stem cell quiescence maintained by p21 cip1/waf1. *Science* **287**:1804-1808.

D'Hondt L, McAuliffe C, Damon J, Reilly J, Carlson J, Dooner M, Colvin G, Lambert J, Hsieh C, Habibian H, Stencel K, and Quesenberry PJ (2004) Circadian variations of bone marrow engraftability. *J Cell Phyisol* **200**:63-70.

Enan E, El-Sabeawy F, Scott M, Overstreet J, and Lasley B (1998) Alterations in the growth factor signal transduction pathways and modulators of the cell cycle in endocervical cells from macaques exposed to TCDD. *Toxicol Appl Pharmacol* **151**:283-293.

Fine JS, Silverstone AE, and Gasiewicz TA (1990) Impairment of prothymocyte activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **144**: 1169-76.

Fu L, Pelicano H, Liu J, Huang P, and Lee CC (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111**:41-50.

Haack M, Pollmacher T, and Mullington JM (2004) Diurnal and sleep-wake dependent variations of soluble TNF- and IL-2 receptors in healthy volunteers. *Brain Behav Immun* **18**:361-367.

Hahn ME (2002) Aryl hydrocarbon receptors: diversity and evolution. *Chem Biol Interact* **141**:131-160.

Huang P, Ceccatelli S, and Rannug A (2002) A study on diurnal mRNA expression of CYP1A1, AhR, Arnt, and Per2 in rat pituitary and liver. *Environ Toxicol Pharmacol* **11**:119-126.

IARC (1997) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Polychlorinated Dibenzo-para-dioxins and Polychlorinated Dibenzofurans. Lyon, France. *IARC Monogr Eval Carcinog Risks Hum* **69**.

Jetmore A, Plett PA, Tong X, Wolber FM, Breese R, Abonour R, Orschell-Traycoff CM, and Srour EF (2002) Homing efficiency, cell cycle kinetics, and survival of quiescent

and cycling human CD34(+) cells transplanted into conditioned NOD/SCID recipients. *Blood* **99**:1585-1593.

Kolluri SK, Weiss C, Koff A, and Gottlicher M (1999) P27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells. *Genes Dev* **13**:1742-1753.

Kondo M, Weissman IL, and Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**:661-672.

Laiosa MD, Wyman A, Murante FG, Fiore NC, Staples JE, Gasiewicz TA, and Silverstone AE (2003) Cell proliferation arrest within intrathymic lymphocyte progenitor cells causes thymic atrophy mediated by the aryl hydrocarbon receptor. *J Immunol* **171**:4582-4591.

Miller JD, Settachan D, Frame LT, and Dickerson RL (1999) 2,3,7,8-Tetrachlorodibenzo-p-dioxin phase advances the deer mouse (*Peromyscus maniculatur)* circadian rhythm by altering expression of clock proteins. *Organohal Comp* **42**:23-28.

Murante FG, and Gasiewicz TA (2000) Hemopoietic progenitor cells are sensitive targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin in C57Bl/6J mice. *Toxicol Sci* **54**:374-383.

Penas EM, Cools J, Algenstaedt P, Hinz K, Seeger D, Schafhausen P, Schilling G, Marynen P, Hossfeld DK, and Dierlamm J (2003) A novel cryptic translocation t(12;17)(p13;p12-p13) in a secondary acute myeloid leukemia results in a fusion of the ETV6 gene and the antisense strand of the PER1 gene. *Genes Chrom Canc* **37**:79-83.

Perpoint B, Le Bousse-Kerdiles C, Clay D, Smadja-Joffe F, Depres-Brummer P, Laporte-Simitsidis S, Jasmin C, and Levi F (1995) In vitro chronopharmcology of recombinant mouse IL-3, mouse GM-CSF, and human G-CSF on murine myeloid progenitor cells. *Exp Hematol* **23**:362-368.

Pollenz RS (2002) The mechanism of Ah receptor protein down-regulation (degradation) and its impact on Ah receptor-mediated gene regulation. *Chem Biol Interact* **141**:41-61.

Richardson VM, Santostefano MJ, and Birnbaum LS (1998) Daily cycle of bHLH-PAS proteins, Ah receptor and Arnt, in multiple tissues of female Sprague-Dawley rats. *Biochem Biophys Res Commun* **252**:225-231.

Sakai R, Kajiume T, Inoue H, Kanno R, Miyazaki M, Ninamiya Y, and Kanno M (2003) TCDD treatment eliminates the long-term reconstitution activity of hematopoietic stem cells. *Toxicol Sci* **72**:84-91.

Smaaland R, Laerum O, Southern R, Stevold O, Bjerkners R, and Lote K (1992) Colony-forming units granulocyte/macrophage and DNA synthesis of human bone marrow are circadian stage dependent and show co-variation. *Blood* **79**:2281-2287.

Staples JE, Murante FG, Fiore NC, Gasiewicz TA, and Silverstone AE (1998) Thymic alterations induced by 2,3,7,8tetrachlorodibenzo-p-dioxin are strictly dependent on aryl hydrocarbon receptor activation in hemopoietic cells. *J Immunol* **160**:3844-3854.

Stevold O, and Laerum OD (1988) Multipotent stem cell (CFU-S) numbers and circadian variations in aging mice. *Eur J Haematol* **41**:230-236.

Stevold O, Laerum O, and Riise T (1988) Age-related differences and circadian and seasonal variations of myelopoietic progenitor cell (CFU-GM) numbers in mice. *Eur J Haematol* **40**:42-49.

Takane H, Ohdo S, Baba R, Koyanagi S, Yukawa E, and Higuchi S (2002) Relationship between 24-hour rhythm in antiviral effect of interferon-β and interferon-α/β receptor expression in mice. *Jpn J Pharmacol* **90**:304-312.

Takane H, Ohdo S, Yamada T, Koyanagi S, Yukawa E, and Higuchi S (2001) Relationship between diurnal rhythm of cell cycle and interferon receptor expression in implanted-tumor cells. *Life Sci* **68**:1449-1455.

Thurmond TS and Gasiewicz TA (2000) A single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin produces a time- and dose-dependent alteration in murine bone marrow B-lymphocyte maturation profile. *Toxicol Sci* **58:** 88-95.

Tsinkalovsky O, Rosenlund B, Laerum OD, and Eiken HG (2005) Clock gene expression in purified mouse hematopoietic stem cells. *Exp Hematol* **33**:100-107.

van Grevenynghe J, Bernard M, Langouet S, Le Berre C, Fest T, and Fardel O (2005) Human CD34-positive hematopoietic stem cells constitute targets for carcinogenic polycyclic aromatic hydrocarbons. *J Pharmacol Exp Ther* **314**:693-702.

Williamson MA, Gasiewicz TA, and Opanashuk LA (2005) Aryl hydrocarbon recptor expression and activity in cerebellar granule neuroblasts: implications for development and dioxin neurotoxicity. *Toxicol Sci* **83**:340-348.

Wood PA, Hrushesky WJ, and Klevecz R (1998) Distinct circadian time structures characterize myeloid and erythroid progenitor and multipotential cell clonogenicity as well as marrow precursor proliferation dynamics. *Exp Hematol* **26**:523-533.

Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, and Lee CC (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* **105**:683-94.

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Fig. 1. TCDD disrupts the circadian rhythms of the numbers of antigenically-defined (LSK) hematpoietic stem cells. Bone marrow cells isolated from control or TCDD-treated animals (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Flow cytometry was performed as described in Materials and Methods. The data shown are representative of 3 separate experiments. Data are presented as mean values ± S.E. There was a treatment-dependent difference for this pattern (ANOVA p value < 0.001), and * indicates statistically different ($p < .05$) from the vehicle-treated value at the same time point.

Fig. 2. TCDD disrupts the circadian rhythms of the numbers of antigenically-defined myeloid progenitors. Bone marrow cell were isolated from control (•) and TCDD-treated (o) animals (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Flow cytometry was performed as described in Materials and Methods. A) CMP. B) GMP. C) MEP. The data shown are representative of 3 separate experiments. Data are presented as mean values ± S.E. There was a treatment-dependent effect for CMP, GMP and MEP (all ANOVA p values < 0.001), and * indicates statistically different ($p < .05$) from the vehicle control value at the same time point.

Fig. 3. TCDD disrupts the circadian rhythms of the numbers of functionally-defined progenitors. Bone marrow cells were isolated from control (•) and TCDD-treated (o) mice (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5

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after treatment. Colony-forming assays were performed as described in Materials and Methods. A) HPP-CFC. B) CFU-GEMM. C) CFU-GM. The data shown are representative of 2 separate experiments. Data are presented as mean values ± S.E. ANOVA p values were 0.14, 0.07, and 0.09 for HPP-CPC, CFU-GEMM, and CFU-GM, respectively.

Fig. 4. TCDD disrupts the circadian rhythm of relative LSK bone marrow cell quiescence. Bone marrow cells were isolated from control and TCDD-treated mice (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 following treatment. Cell cycle analysis was performed as described in Materials and Methods. The data presented are the percent of total number of LSK cells in quiescence (G_0) ± S.E. and is representative of 2 separate experiments. There was a treatment-dependent difference for this pattern (ANOVA p value < 0.001), and * indicates statistically different ($p < .05$) from the vehicle control value at the same time point.

Fig. 5. TCDD disrupts the circadian rhythms of Per1/Per2 mRNA levels. Lin^- $Sca\text{-}1^+$ bone marrow cells were isolated from control (•) and TCDD-treated (o) animals (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 at day 5 following treatment.. Total RNA was isolated as described in Materials and Methods. Quantitative RT-PCR was performed using primer/probe sets specific to A) mPer1 and B) mPer2. Both mPer1 and mPer2 demonstrated treatment-dependent differences in the diurnal patterns (ANOVA p values were 0.049 and < 0.001, respectively), and * indicates statistically different ($p < .05$) from the vehicle control value at the same time point.

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Fig. 6. AhR and Arnt mRNA levels in Lin$^-$ Sca-1$^+$ bone marrow cells are governed by circadian rhythms. Bone marrow cells were isolated from control mice (n = 6 per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 following treatment of animals with vehicle (olive oil). Total RNA was isolated as described in Materials and methods. Quantitative RT-PCR was performed using primer/probe sets specific to AhR (•) and Arnt (o).

Fig. 7. TCDD up-regulates AhRR mRNA in Lin$^-$ Sca-1$^+$ bone marrow cells. Bone marrow cells were isolated from control and TCDD-treated mice (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Total RNA was isolated as described in Materials and Methods. Quantitative RT-PCR was performed using primer/probe sets specific to AhRR. There was a treatment-dependent difference in this pattern (ANOVA p value < 0.001), and * indicates statistically different ($p < .05$) from the vehicle control value at the same time point.

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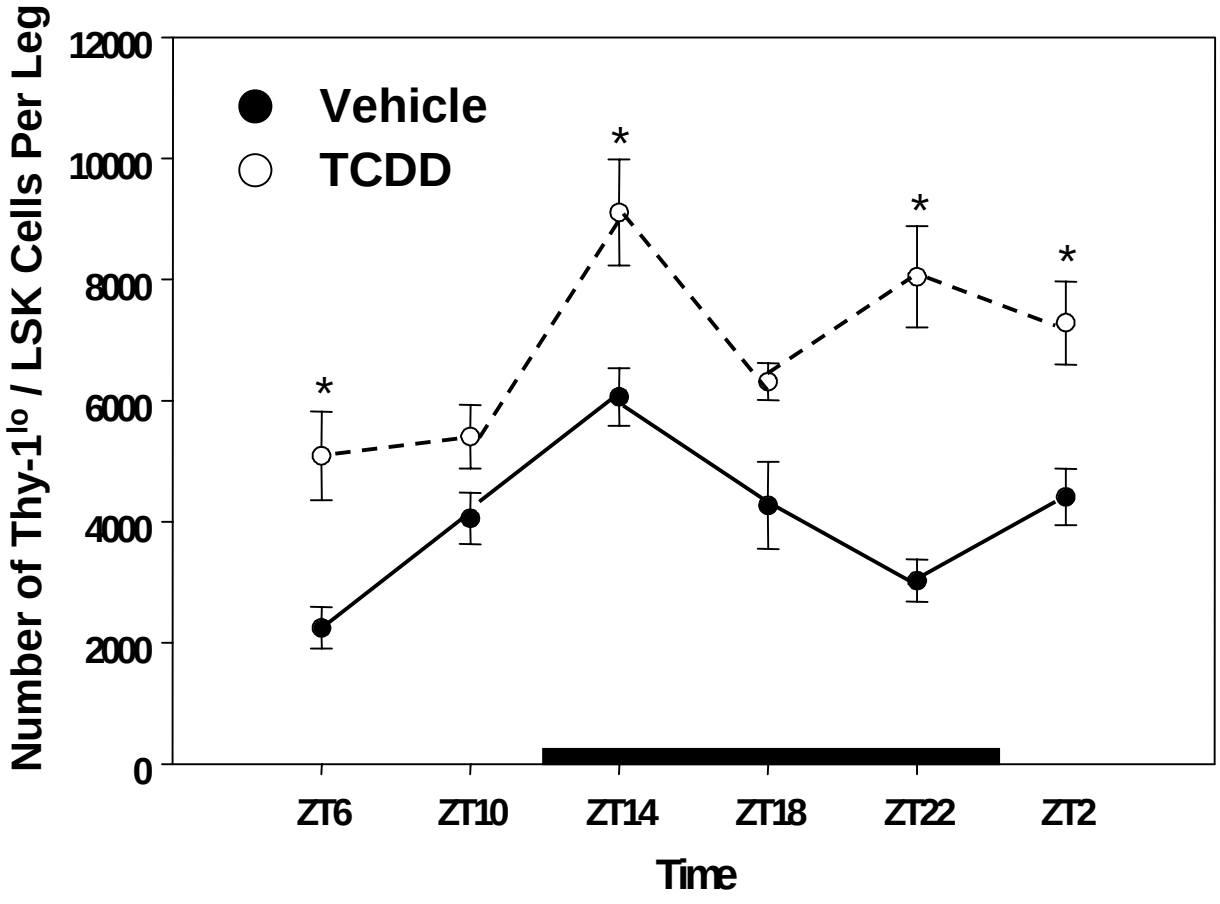

Fig. 1

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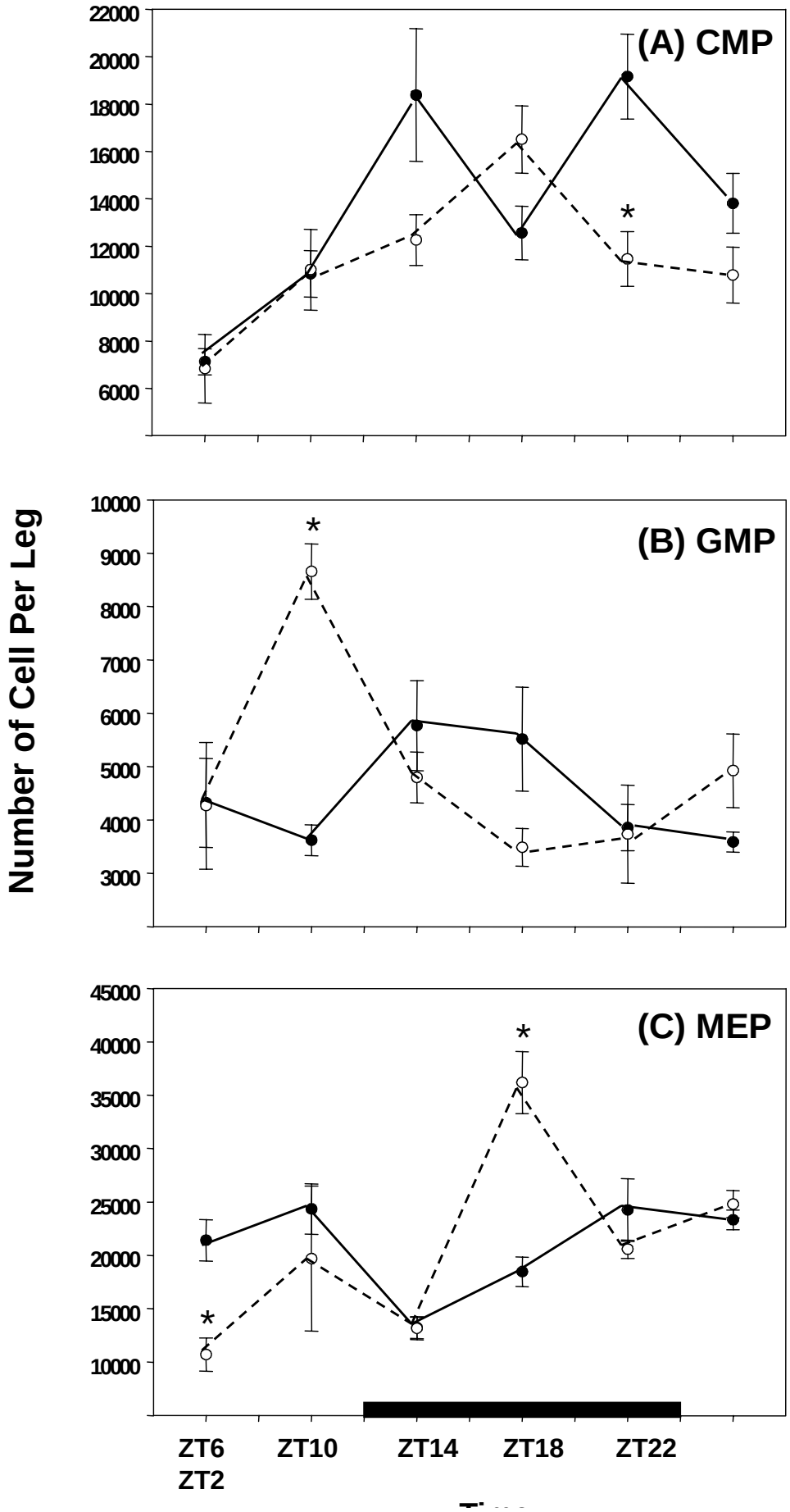

Fig. 2

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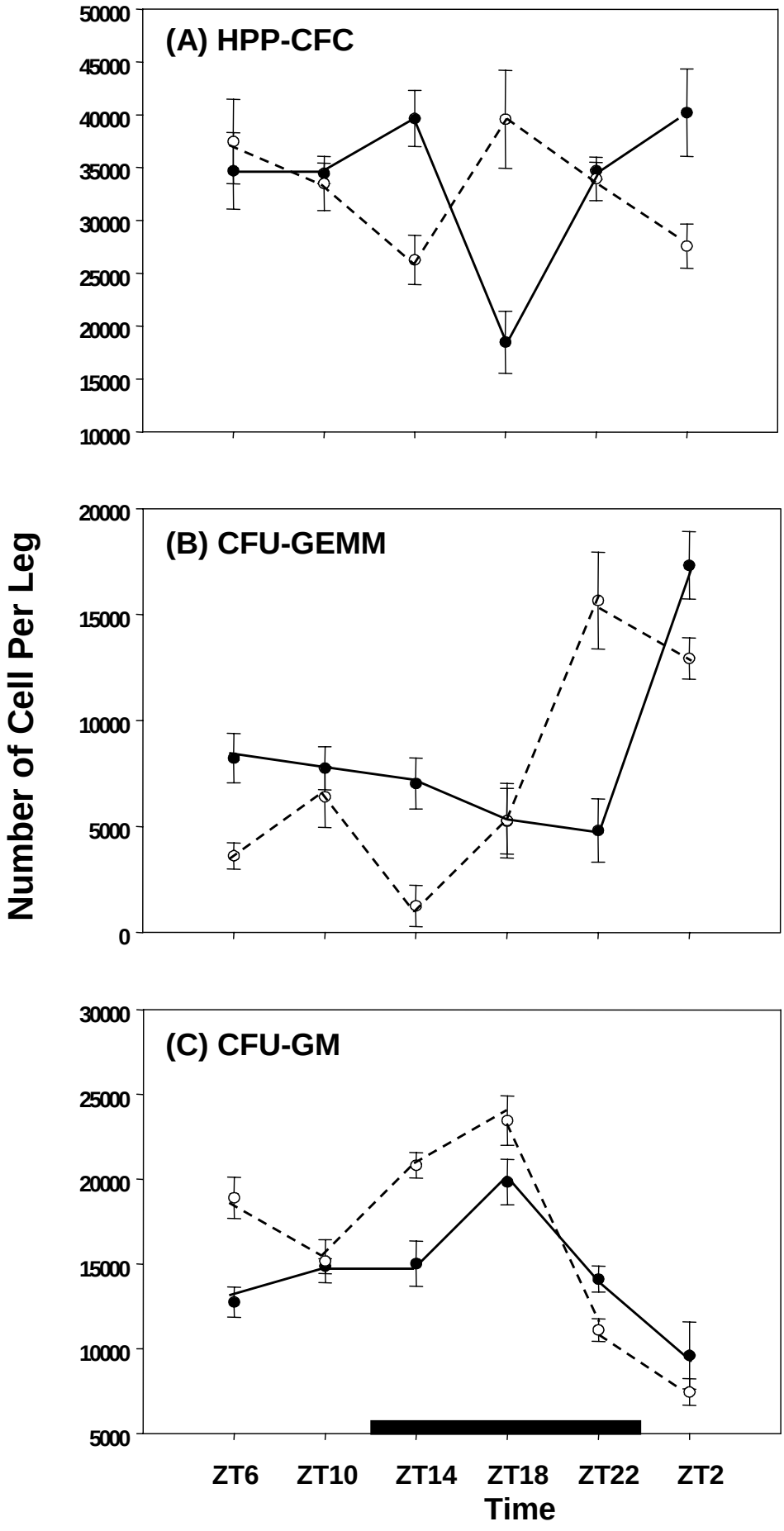

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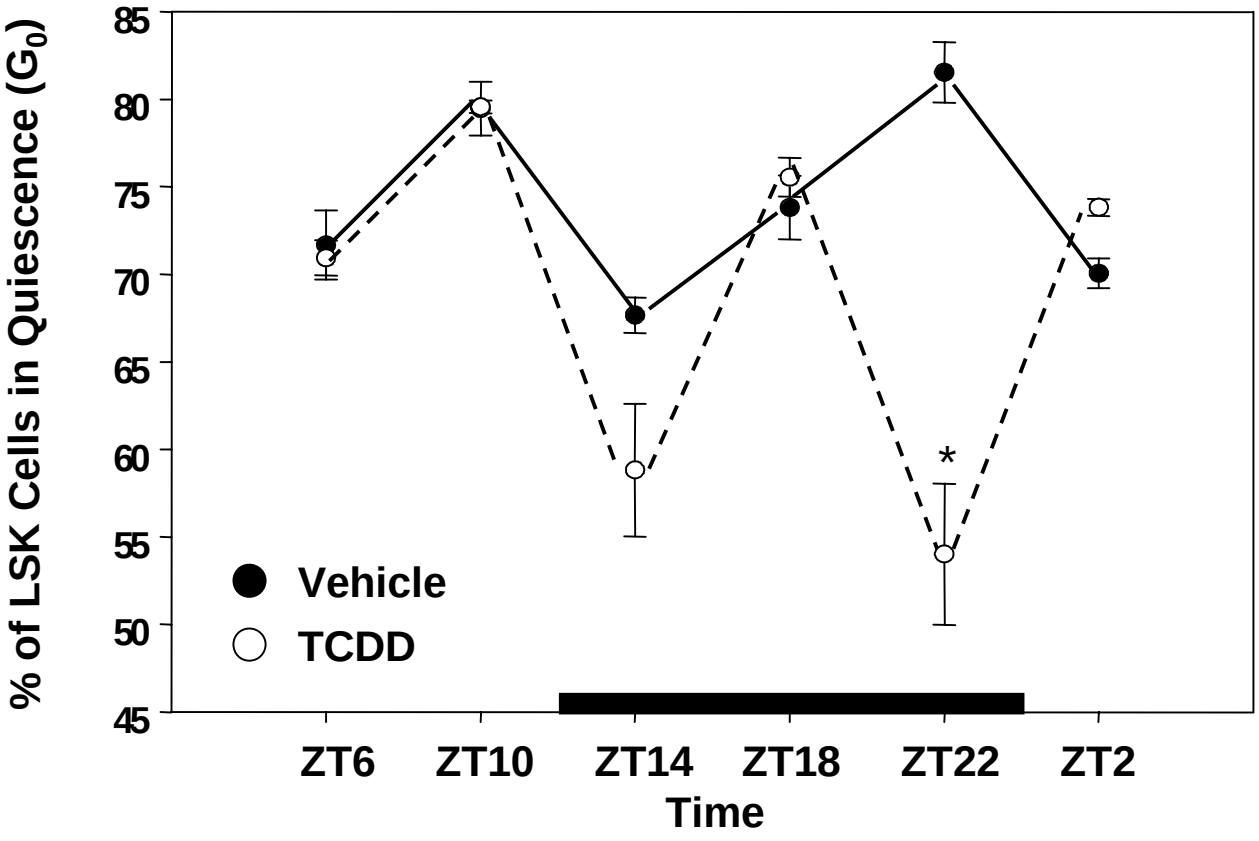

Fig. 4

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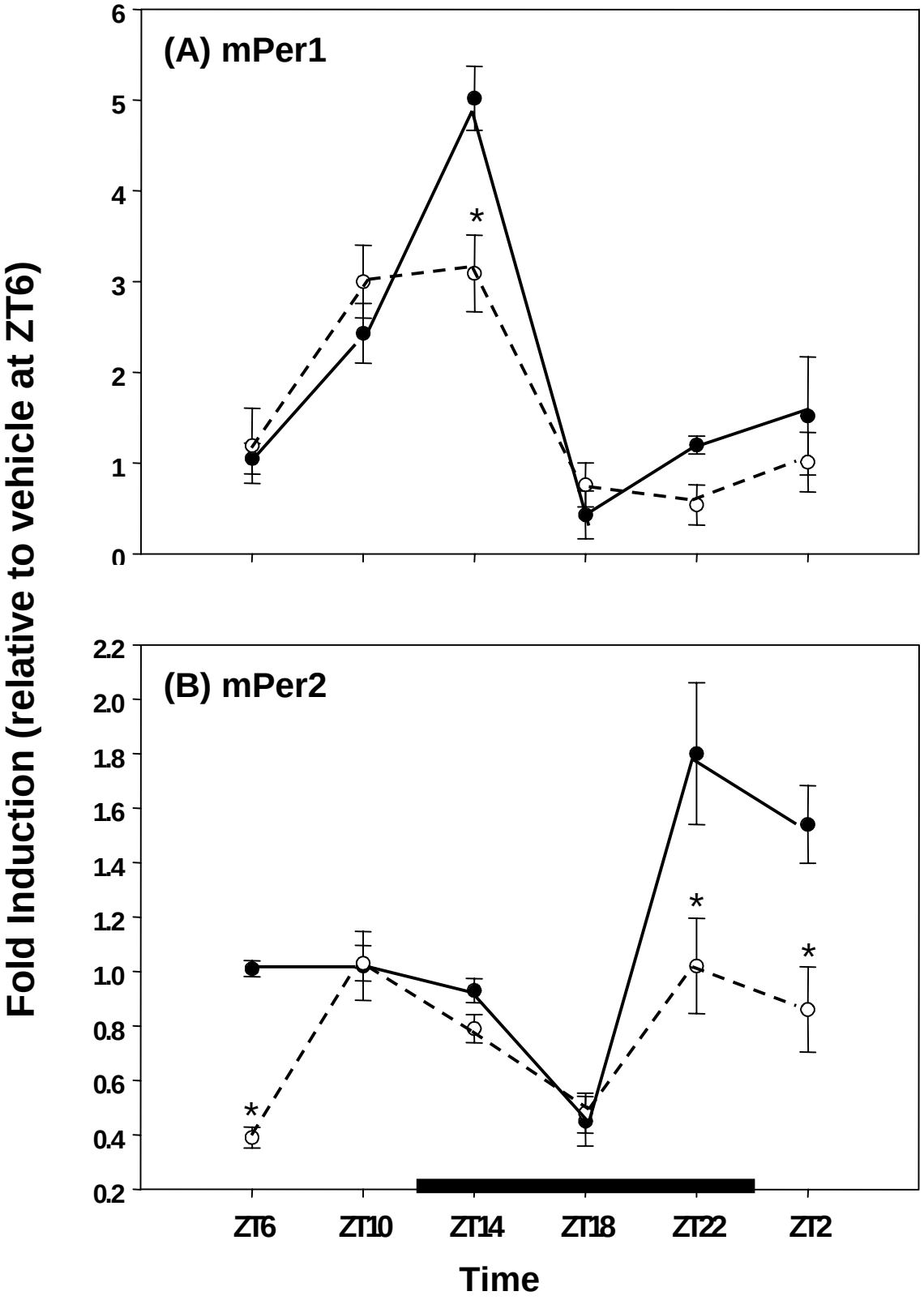

Fig. 5

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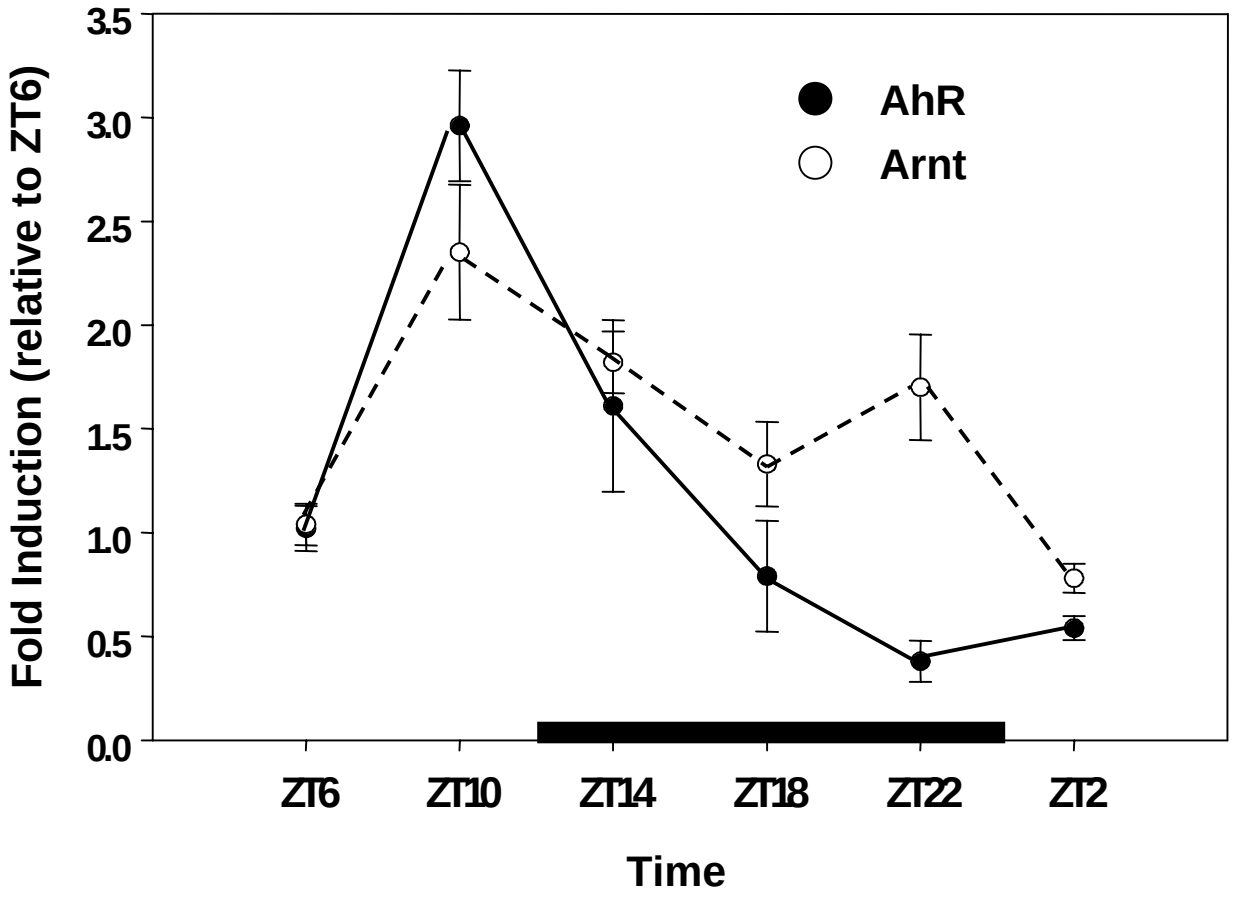

Fig. 6

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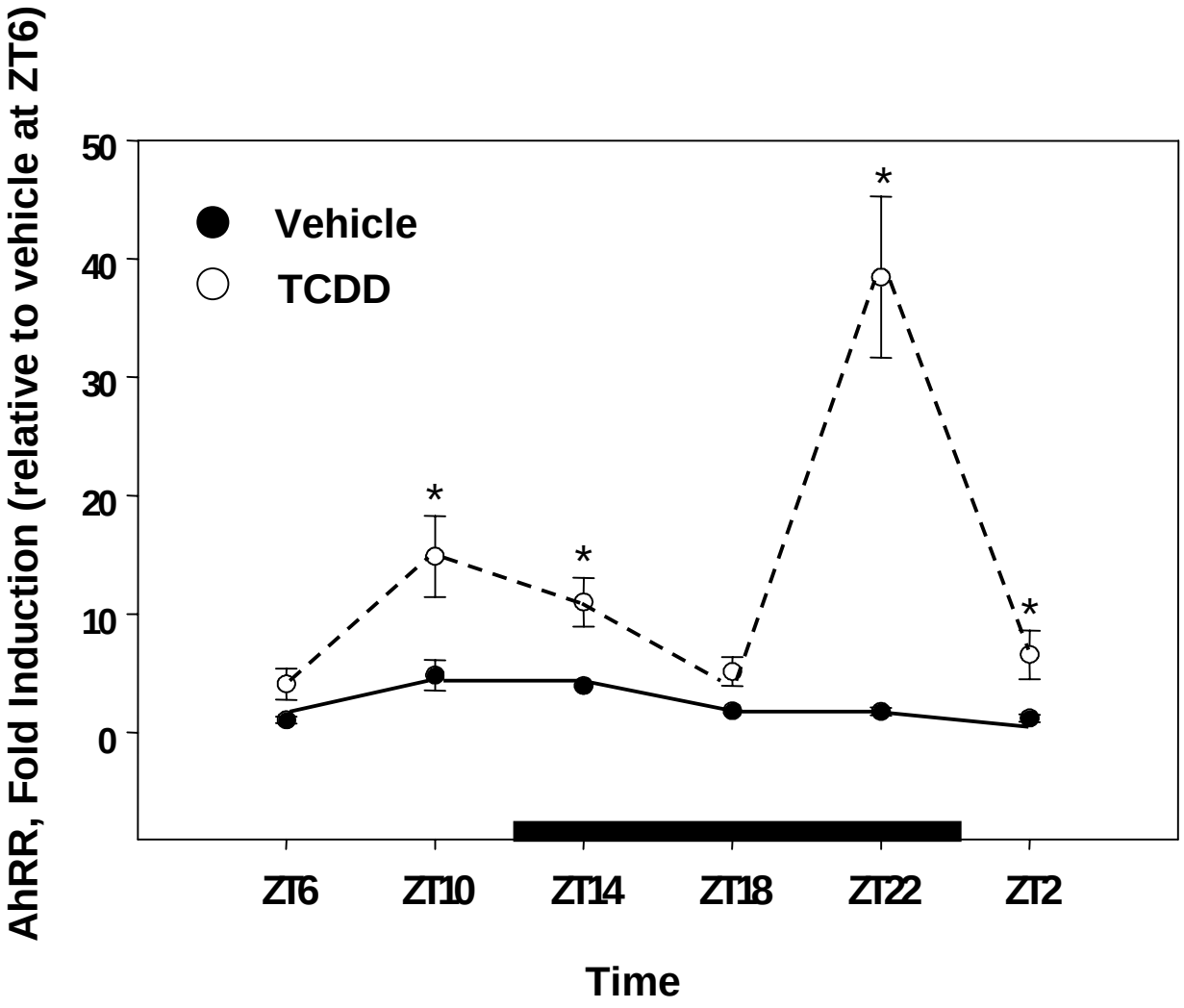

Fig. 7