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Effects of a single exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on macro- and microstructures of feeding and drinking in two differently TCDD-sensitive rat strains ☆

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ABSTRACT

In rats, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes anorexia that may lead to fatal wasting but has hitherto been poorly characterized. Therefore, we studied in-depth feeding and drinking behaviors of TCDD-sensitive L–E rats for 5 (100 μg/kg; lethal dose) or 10 (10 μg/kg; sublethal) days and of TCDD-resistant H/W rats for 14 (100 or 1000 μg/kg; both sublethal) days postexposure to TCDD. The 1000-fold higher resistance of H/W rats to acute lethality of TCDD results from a mutation in their AH receptor (AHR). We split days into four (morning, daytime, evening, and night) or two (light/dark) circadian periods and took the repeated nature of the data into account. In L–E rats at 100 μg/kg, the feed intake dropped precipitously, due to reduced meal sizes. In H/W rats, the hypophagia remained moderate and stemmed from a reduced meal frequency. While the suppression in L–E rats peaked during the morning (at 100 μg/kg), the main effects in H/W rats were seen during the constant light or dark phases. Furthermore, chronologic data analysis revealed alterations in consecutive feeding and drinking patterns. Thus, striking differences were found between these strains in the timing and structure of consummatory behaviors, suggesting involvement of the AHR in these behaviors.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent compound of the polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/PCDFs). These persistent environmental pollutants, commonly called dioxins, are mostly formed as harmful by-products in thermal, chemical or technical processes. Humans are exposed to dioxins mainly via food (fatty fish, meat, and dairy products), because dioxins are fat-soluble, stable, and accumulative in the food chain. Dioxins differ in their toxic potencies, but share a structural similarity as well as a common mode of action. Exposure to them can cause a variety of toxic effects including cancer, developmental defects, immune toxicity, endocrine disturbances, and hypo- or hypertrophia of target tissues. Furthermore, species and even (sub)strains within species differ widely in sensitivity to these toxic effects and display qualitative differences in responses [reviewed e.g. in (Lindén et al., 2010; Pohjanvirta and Tuomisto, 1994)]. A single exposure may be sufficient to elicit the effects, due to the poor biotransformation and elimination of TCDD in the organism (Gasiewicz and Neal, 1979; Hakk et al., 2009; Pohjanvirta et al., 1990b).

In many experimental animals, TCDD causes a dose-dependent suppression of feed intake which culminates in a wasting syndrome at lethal doses (Pohjanvirta and Tuomisto, 1990a). Anorexia is one of the responses to TCDD, showing variability among animal species and strains. TCDD exposure not only affects total feed consumption—by adjusting it permanently to a lower level—but also elicits specific alterations in feeding behavior (Lensu et al., 2011b, 2011c; Pohjanvirta and Tuomisto, 1990a; Pohjanvirta et al., 1990a; Tuomisto et al., 2000). Apart from those related to feeding, marked behavioral changes are few in adult rats after an acute exposure to TCDD (Sirkka et al., 1992).

What makes the mechanistic studies of TCDD-caused wasting so challenging are the diverse features of this drastic response (for a recent review, see Lindén et al., 2010). For example, exposed rats become hypersensitive to postingestive satiety signals (Pohjanvirta and Tuomisto, 1990a; Pohjanvirta et al., 1991), but are hyposensitive to insulin- or 2-deoxyglucose-elicited feeding (Pohjanvirta and Tuomisto, 1990b; Pohjanvirta et al., 1990a). Their body weight

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(BW) set-point seems to be adjusted to a lower level following exposure, since TCDD-treated rats are capable of defending their lowered BW level against various feeding challenges (Pohjanvirta and Tuomisto, 1990a, 1990b; Seefeld et al., 1984a, 1984b; Tuomisto et al., 1999a). Although the loss of BW results from hypophagia and depletion of energy stores (Christian et al., 1986; Weber et al., 1991), neither force-feeding (Gasiewicz et al., 1980; Tuomisto et al., 1999a) nor obesity or high-energy diet (Tuomisto et al., 1999a) could postpone the time of death following lethal doses of TCDD. Furthermore, wasting is not a consequence of nausea or an alteration in energy metabolism or locomotor activity (Pohjanvirta et al., 1994; Potter et al., 1986; Seefeld et al., 1984a; Seefeld and Peterson, 1984). Even though TCDD is known to affect several physiological mechanisms involved in the maintenance of BW balance and energy homeostasis [reviewed e.g. in (Bock and Köhle, 2006; Lindén et al., 2010; Mandal, 2005; Pohjanvirta and Tuomisto, 1994; Unkila et al., 1995)], the exact mechanistic pathways and biochemical factors underlying the syndrome are still unknown.

In rats, sensitivity to the acute lethality of TCDD is reflected in the severity of the wasting syndrome (Pohjanvirta and Tuomisto, 1994). Therefore, in the present study we utilized two differently TCDD-sensitive rat substrains: Long–Evans (*Turku/AB;* L–E) and Han/Wistar (*Kuopio*; H/W), a rat model exhibiting a 1000-fold difference in sensitivity to the acute lethality of TCDD. At lethal doses of TCDD the sensitive L–E rats rapidly, substantially, and irreversibly reduce feeding; the consequent BW loss leads to death within 2–5 wk. At very high doses of TCDD, the resistant H/W rats also exhibit changes in their feed intake: they either show a fairly mild reduction or undergo a total fast that may last up to 11 d before they resume eating. Even thereafter, the BW of H/W rats lag behind that of their unexposed controls by 5–10% (Pohjanvirta and Tuomisto, 1987, 1990a; Pohjanvirta et al., 1987).

The resistance of H/W rats results primarily from a mutation in the aryl hydrocarbon receptor (AHR); L–E rats have a wild-type AHR (Pohjanvirta et al., 1998). This ligand-activated transcription factor is involved in responses to xenobiotics, and all major effects of TCDD assessed so far have proven to be mediated via binding of TCDD to the AHR. However, in addition to mediating the biological effects of xenobiotics, the AHR is involved in numerous important physiological functions, such as vascularization, regulation of the cell cycle, and maintenance of circadian rhythms (Furness and Whelan, 2009; Mukai et al., 2008; Mukai and Tischkau, 2007). Studies on physiological functions, using TCDD as a tool to activate the AHR, may thus be as important as its use in toxicology studies.

To gain further insight into TCDD-induced hypophagia, we set up an automated monitoring system that allowed us to analyze the feeding and drinking behaviors of TCDD-treated rats continuously for extended periods. Short-term intake analyses or total daily consumptions provide only rough and insufficient information of feeding and drinking behaviors. Furthermore, TCDD exposure alters the circadian rhythms of feeding in rats (Christian et al., 1986; Pohjanvirta and Tuomisto, 1990a, 1990b; Pohjanvirta et al., 1988), but this effect has not been thoroughly characterized. Therefore, the anorectic effects of TCDD were now investigated at the micro- and macrostructural levels, and details of individual feeding and drinking bouts were encompassed. In addition to the nature, magnitude, and number of changes, we determined their timing in relation to the light/dark (L/D) cycle. The findings of this study will shed more light on the TCDD-induced wasting syndrome and aid in orienting subsequent studies of TCDD toxicity with regard to the most appropriate time-points for assays.

2. Material and methods

2.1. Animals, animal facilities, and habituation

In the study, 18 ± 4-wk-old male rats were used. TCDD-sensitive L–E rats [an inbred strain, lethal dose for 50% of exposed animals (LD_{50}) 10–20 μg/kg (Pohjanvirta et al., 1993)] and TCDD-resistant H/W rats [originally outbred but currently a random-bred strain, LD_{50} > 9600 μg/kg (Unkila et al., 1994)] were habituated to daily handling and experimental conditions thoroughly. Throughout the study, all rats were kept in the same room and they were weighed at least three times per week. Before the animals were moved into test cages, they were habituated to eating 45-mg dust-free precision pellets (Bio-Serv®, Frenchtown, NJ, USA) for about 1 wk. They were further accustomed to Habitest® (Coulbourn Instruments, Whitehall, PA, USA) cages for at least a week, and experiments were initiated only when the feeding and drinking of the rats had stabilized at the normal level (number of licks app. 7000 and number of pellets app. 450 per day). Only rats that met these criteria were included in the study.

Both the pellets and tap water were available *ad libitum* throughout the experiments. The energy content of the precision pellets was 3.6 kcal/g. According to the manufacturer, the feed consisted of 21% protein (corresponds to 25% of total energy), 4.8% fat (12% of total energy), and 58% carbohydrate (63% of total energy). In addition, the pellets contained 4% fiber, 7% ash, and less than 10% moisture. The test cage for a single rat (floor size 25 cm × 30 cm, height 30 cm) had a wire-mesh bottom and Plexiglas walls. Autoclaved aspen chips and wooden toys (Tapvei Co., Kaavi, Finland) were given for each rat to enrich the environment. Part of the front wall of the cage was covered with black plastic, providing visual shelter for the animal.

The animal room was controlled for light (12/12 h L/D cycle, lights on at 7 a.m.), temperature (22 ± 2 °C) and humidity (50 ± 20%). The experiments were reviewed and approved of by the Committee for the Welfare of Laboratory Animals of the University of Kuopio and by the Provincial Government. The procedures were conducted in accordance with the Guidelines of the European Community Council directives 86/609/EEC.

In the study, we had two test cages and therefore monitored the rats in pairs. The simultaneously analyzed TCDD-treated and control rats were matched for weight and age. The rats were videotaped for a more detailed analysis of behaviors unrelated to feeding and drinking (data not shown here). Due to the videotaping, the room was illuminated with a red lamp (darkroom safelight bulb, Philips PF712E, 15 W, wavelength > 630 nm, intensity 7 lumen) during darkness. However, the lamp was directed away from the cages to keep the lighting as dim as possible to produce just enough illumination required by the video cameras. The number of pairs was six for H/W rats exposed to 100 μg/kg TCDD; in all other groups it was seven. Due to errors in data storage, one control L–E rat had to be excluded from the study (control rat for an animal at a dose of 100 μg/kg). On the day of exposure (day 0), the control L–E rats weighed 322.9 ± 9 g (mean ± SE; n = 13) and TCDD-exposed L–E rats 325.7 ± 9 g (n = 14). The control H/W rats weighed 416.0 ± 12 g (n = 13) and TCDD-exposed H/W rats 420.7 ± 11 g (n = 13) (Suppl. Fig. 1).

2.2. Measurements with automated system

Feeding and drinking data were continuously collected with the Coulbourn Habitest® system. It was controlled by Graphic State 3.02 software (Coulbourn Instruments, Whitehall, PA, USA), which handles the data in a binary form. In this system, two nose-poke holes are situated on the same wall of the cage and both holes are equipped with infrared light sources and sensors connected to a computer. The infrared light beam in the feeder is interrupted only when a feed pellet is removed from the pellet tray, thereby avoiding inadvertent beam breaks by the tail and distortion of the data. The feeder delivered a single pellet automatically after the rat had removed one and the computer recorded the event. The water bottle was fixed to the cage in such a way that by each lick the rat's tongue interrupted the infrared light beam; each interruption was counted. The time unit for

these measurements was set at 100 ms; thus, the equipment was able to distinguish events with lags longer than 0.1 s. The same lag was used for both the lickometer and the feeder. Each time any of the feeders or lickometers was activated, the time of the event was recorded in milliseconds (ms) with reference to the start of the measurement. The stored binary data were further processed into feeding or drinking episodes with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Since the system was unable to record wasted pellets, we collected spilled pellets and measured them daily to determine the exact amount eaten for each rat. However, we did not take into account the spilled feed in feeding microstructure analysis, because it was impossible to define afterwards the exact time and the specific meal during which the spillage had occurred. The volume of spilled water was also monitored, but it was negligible.

2.3. TCDD treatment

After baseline measurements for several days, the rats were treated either with TCDD (UFA-Oil Institute, Ufa, Russia; intragastric [ig] administration) or the vehicle, corn oil (Sigma-Aldrich, C8267, St. Louis, MO, USA). The TCDD was over 99% pure as confirmed by gas chromatography–mass spectrometry (Vartiainen et al., 1995; Viluksela et al., 1998) and was dissolved in corn oil, as previously described (Simanainen et al., 2002). The administration volume for both was 4 ml/kg ig, and the amount of TCDD in the solution was 2.5, 25, or 250 μg/ml, depending on the dose. After TCDD exposure, the rats were monitored for 5–14 d. The exposure occurred at noon on day 0 (depicted with a downward arrow in the figures).

2.4. Data analysis and definitions

The patterns of feeding and drinking behaviors were compared across treatments, measuring days and subsections of time. The days were split into diurnal periods: morning, daytime, evening, and night period. Using these four circadian phases enabled us to obtain more information on the L/D transitions compared with the traditional L/D distribution. Morning and evening were 5 h periods around the light changes: from 5:00 a.m. to 9:59 a.m. and from 5:00 p.m. to 9:59 p.m., respectively. Daytime was the fully illuminated period (10 a.m.–4:59 p.m.) and night the period of total darkness (10 p.m.–4:59 a.m.). However, for some days (0, 2, 5, and 10 d after the exposure) we also analyzed some variables of the data according to the traditional lighting rhythm (illuminated period between 7 a.m. and 7 p.m. and darkness between 7 p.m. and 7 a.m.).

Although the data were gathered from pairs (control and TCDD-treated rat), the external conditions appeared to be constant across the pairs, enabling us to combine the control data. The pooled data of controls for both strains were used in the analysis. In statistical analysis, the repeated nature of the data was taken into account. Furthermore, the statistical models used are not sensitive to differences in group size and therefore the uneven number of rats in the groups did not hamper the analysis.

The data were subjected to several types of analysis of feeding and drinking. Initially, the data were split according to the type of behavior, and the feeding and drinking behaviors were analyzed separately. For the analyses of meals or drinking bouts, the feeding or drinking events were aggregated into episodes. A single meal was defined as an event including all instances of two consecutive pellets having a lag shorter than 5 min between them. This time limit was chosen, based on the temporal patterns of feeding: 97% of all pellets eaten were taken up in bouts in which the lag between pellets fell within 5 min. The same 5 min lag (between tongue licks) was used for the definition of drinking episodes, with more than 99% of the total number of licks residing within this range. However, to determine the total feed and water consumption and their circadian variation, episodic data of meals and drinking bouts were pooled for each rat. The total numbers of meals and drinking bouts within 1 d, total time used for eating or drinking, and total consumptions were calculated from the pooled data individually for each rat. The corresponding values were also calculated as per the diurnal period of the day. In the analyses, missing events (if the rats were not drinking or eating during a circadian period) were encoded as zero values.

In all, the parameters used in the present study were the following: meal size (g) or number of licks in a drinking bout, duration of a meal or a drinking bout (min), time to the following meal or a drinking bout (interbout interval, min), satiety or thirst (time from the previous meal or drinking bout to the following divided by the amount of the previous consumption), and feeding or drinking rate (calculated as the amount eaten or drunk divided by the duration of an episode). The drinking data are shown as number of licks because the volume of each lick is unknown.

Finally, we also analyzed the behavioral changes in chronological order. For this analysis, the split data of feeding- and drinking-related episodes were combined, again separately for each rat. This allowed us to evaluate episodic behavior (how meals and drinking bouts followed each other). The time lags between consecutive meals and drinking bouts as well as the meal-to-drinking bout lags (or vice versa) were calculated. To calculate the total number of events for each rat and for each day (or for a period of the day), the chronological data on feeding and drinking bouts were further pooled rat-wise. Nevertheless, in the combined data the feeding and drinking events were not redefined. Hence, the combined data of feeding and drinking include meals and drinking bouts as separate entities, although there could have been lags shorter than 300 s between a meal and a drinking bout or vice versa.

2.5. Statistical analysis

Statistical data analyses were performed with the SPSS 17.0 software. For continuous responses a linear mixed model and for count responses generalized linear Poisson model with generalized estimating equations (GEEs) were used (Zeger and Liang, 1986). With these models, repeated daily measures of feeding or drinking parameters (described above) were analyzed by accounting for the dependence of the observations from the same rat. Autoregressive and exchangeable correlation structures were used accordingly to model the dependence of the observations of feeding and drinking on the dose of TCDD (group), on the measuring day (relative to exposure), and on the circadian period, both in linear mixed models and in GEE models. Type III tests of fixed effects with Sidak's adjustment for multiple comparisons were used. The level of statistical significance was set at a P-value of ≤ 0.05.

The data are shown as mean ± SE. In the figures, only significant differences between the TCDD-treated and control rats are depicted, and the effects of measuring day or circadian period among the groups are not shown. However, the preexposure levels were mostly equal among the groups within each strain and circadian period and the day-to-day variation similar.

3. Results

3.1. General effects of TCDD exposure on food intake, feed spillage, and body weight

While the control rats of both strains gained weight in a similar manner over the experimental period, their TCDD-treated counterparts lost weight (Fig. 1 and Suppl. Fig. 1). In L–E rats, the weight loss induced by TCDD was swift at the ultimately lethal dose of 100 μg/kg, reaching about 15% by day 5 (end of observation). At 10 μg/kg it was less dramatic (app. 10% by day 11). Both of the high, but sublethal, doses used in the H/W rats (100 and 1000 μg/kg) resulted in an initial

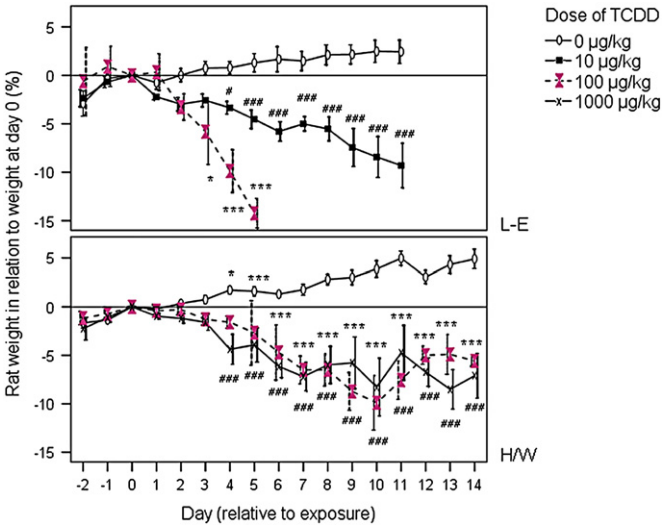

Fig. 1. TCDD exposure diminished body weights of male L–E rats and H/W rats in relation to each rat's own weight on the exposure day (Day 0). Treatments are shown on the upper right corner of the panels, the symbols represent mean values and the error bars show the SE. Group sizes were the following: controls (both strains) n = 13; 10 µg/g L–E, n = 7; 100 µg/kg L–E, n = 7; 100 µg/kg H/W: n = 6; 1000 µg/kg H/W, n = 7. For clarity, only differences between the controls and TCDD-treated groups are shown. Differences were statistically assessed by the linear mixed model (*** = $p<0.005$; ** = $0.01>p\geq0.005$; * = $0.05\geq p\geq0.01$, dose 100 µg/kg and $^{\#\#\#}$ = $p<0.005$; $^{\#\#}$ = $0.01>p\geq0.005$; $^{\#}$ = $0.05\geq p\geq0.01$, doses 10 and 1000 µg/kg).

BW loss for about 1 wk, followed by stabilization of BW thereafter at a level that was app. 10% lower than that at the exposure.

The total daily feed consumption of the rats paralleled the BW change in L–E rats (Fig. 2A). At 100 µg/kg, feed consumption dropped to a very low level (~4 g) by day 5, but the rats did not entirely stop eating. The lower dose decreased intake by ~30% during the latter half of the observation period. However, it is important to note that L–E rats exposed to 100 µg/kg TCDD not only progressively diminished their feed intake but also spilled the feed increasingly on a daily basis (Fig. 2C,D). On the final day the spilled amount was over 30%. After the sublethal dose of 10 µg/kg, feed spillage did not increase significantly.

The TCDD-resistant H/W rats also diminished feed intake down to 50% by day 5 and increased feed spillage following both high doses of TCDD (Fig. 2). Approximately 1 wk after TCDD exposure, the H/W rats began to gradually eat more and spill less, resulting in an increase in the total amount eaten (Fig. 2A,D). Still, they never fully reached the control level in the course of the study.

To take into account the TCDD-caused BW loss in feeding, the metabolic weight $[BW(kg)]^{0.67}$ was calculated and the feed consumption was related to it (Donhoffer, 1986; Feldman and McMahon, 1983). However, this procedure did not appreciably modify the outcome (Fig. 2B).

3.2. Effects of TCDD exposure on diurnal distribution of feeding

Control rats of both strains exhibited continuous, uninterrupted patterns in their circadian feeding rhythms throughout the study (Fig. 3). Both strains consumed 30–45% of their daily intake during each of the two periods of major feeding activity, evening and night (Fig. 3C,D). In the L–E strain the morning hours were also a time of high feeding activity, during which they ate twice as much as did the H/W rats. The converse was true during night and daytime (Fig. 3A,B).

In L–E rats, TCDD (particularly at higher doses of 100 µg/kg) affected feeding most severely in the morning, which was the only period to also show a decrease in relative terms (Fig. 3A,C). The impact was delayed on night and evening intake (in grams of feed) and did not involve daytime intake (g) at all (Fig. 3A). In stark contrast to this pattern, the feed intake (g) in H/W rats was depressed most (down to zero-intake level) during the day hours, especially at lower doses (100 µg/kg). Therefore, the proportional intake tended to increase in the evening (both doses) or, sporadically, during the night (100 µg/kg; Fig. 3B,D).

3.3. Circadian microstructure of feeding

In L–E rats, the lethal dose of TCDD (100 µg/kg) reduced meal size in all circadian periods except for daytime. The meal number was clearly less affected, being decreased only during the morning hours of the last 2 d of observation (days 4 and 5) for this dose. Interestingly, feed intake was preferentially affected in the morning by this high dose of TCDD, since all three variables measured (number, size, and duration of meals) were simultaneously depressed in the morning hours only (Fig. 4A,C,E). At 10 µg/kg, the only change was a delayed and slight decrease in the number of meals during daytime (Fig. 4A).

The H/W rats again displayed a conspicuously different pattern of responses. First, the depressing effect of TCDD in them predominantly involved the number of meals, which diminished in all circadian periods (Fig. 4B). Second, the recovery phase after the first week postexposure was manifested by markedly increased meal sizes, again involving all periods indiscriminately.

Meals lasted longer in the control L–E rats than in their H/W counterparts during all daily phases (Fig. 4E,F). Save for the morning hours in L–E rats treated with 100 µg/kg, the TCDD treatment tended to prolong meal duration in both strains. This feature became highly prominent in H/W rats during their recovery phase, so that their meals then lasted even longer than those of L–E rats.

TCDD had little effect on the total time the rats spent eating during the first week postexposure, with the most notable alterations being subtle reductions in the morning or daytime intakes of L–E and H/W rats, respectively, at the 100-µg/kg dose (Fig. 5A,B). Thereafter, all but the morning intake times tended to increase in H/W rats. The satiety index was elevated, due to prolonged intermeal intervals by TCDD, especially during the morning and night hours in H/W rats (Fig. 5C,D). In eating rates, a dichotomous response was seen, depending on meal size: for meals larger than 0.54 g (*i.e.* more than 12 pellets; smallest meals excluded), there was a clear downward tendency after TCDD treatment in both strains and in all circadian phases. For all meals, in contrast, the situation was reversed in L–E rats at 100 µg/kg and in H/W rats at both dose levels (Suppl. Fig. 2).

To complement the data, some major consummatory variables were further analyzed over the entire illuminated and dark periods (12/12 h) on days 0, 2, 5, and 10 (Suppl. Table 1). This approach confirmed the strain-specific depressive effect of TCDD on either meal size (L–E) or meal frequency (H/W). However, while the meal size was decreased in L–E rats in both lighting phases (at 100 µg/kg), the effect of TCDD on meal number in H/W rats varied, depending on the dose: at 100 µg/kg it was reduced during the light hours but at 1000 µg/kg during the dark hours. Meals lasted longer in the recovery phase of H/W rats, and during daytime on day 10 at 10 µg/kg in L–E rats. The 100 µg/kg dose of TCDD to L–E rats increased the ratio of water vs. feed intake at both the light and dark phases on day 5 (Suppl. Table 1). In H/W rats, the change in this variable was a similar but transient elevation at 1000 µg/kg during the dark phase on days 2 and 3. At 100 µg/kg the ratio elevated during the dark phase on day 8 and during daytime on day 9 (data not shown).

3.4. Effects of TCDD exposure on drinking macro- and microstructure

In control L–E rats, most diurnal drinking occurred evenly in the evening, night, and morning phases, while H/W rats drank about 50–60% of their daily water consumption during the night (Fig. 6E,F). The H/W rats had fewer licks per episode than the L–E rats (Fig. 7E,F), but

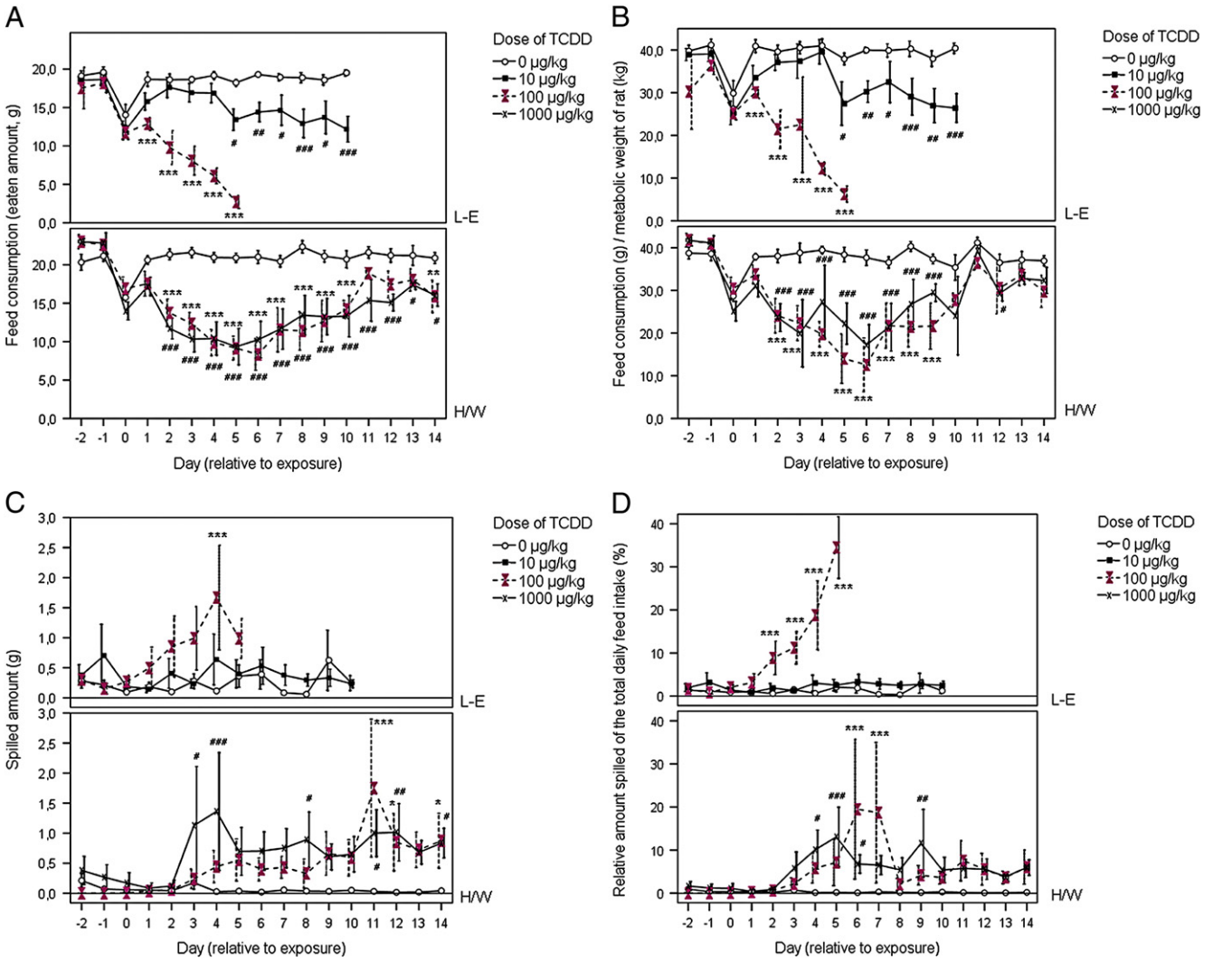

Fig. 2. TCDD exposure diminished feeding and increased feed spillage in L-E and H/W rats. Panel A depicts total eaten amount (g), spilled amount deducted. To take into account the alterations in body weight, metabolic weights [body weight (kg)$^{0.67}$] were calculated: panel B shows feed intake in relation to the metabolic weights (g/kg$^{0.67}$) of rats. The amount of feed spillage (g) increased following exposure in both strains (C). After the high 100 µg/kg dose of TCDD L–E rats spilled about one third of the feed which they took from the feeder (D). Group sizes and statistically significant differences from controls are as in Fig. 1. Treatments are shown on the upper right corner of the panels, the symbols represent mean values and the error bars show the SE.

during the night hours they had more than twice as many drinking bouts as did the L–E rats (Fig. 7A,B).

The TCDD treatment tended to diminish total daily water consumption in both strains (Fig. 6A,B). In L–E rats, the dose of 100 µg/kg reduced total and episode-based water consumption in the night and morning phases (Fig. 6C, 7E). Overall, this lethal dose shifted diurnal drinking from morning to daytime (Fig. 6E), during which phase the number of drinking bouts actually increased (Fig. 7A). At the 10-µg/kg dose, both water consumption per episode (Fig. 7E) and the number of episodes (Fig. 7A) tended to diminish, but the diurnal distribution of drinking activity remained almost unaffected (Fig. 6E).

In the H/W strain, there was a conspicuous drop in water consumption during the night hours (Fig. 6D), which was a consequence of both a lessened amount drunk per episode (Fig. 7F) and a tendency to a decreased number of episodes (Fig.7B). Daytime drinking was practically unaffected by TCDD (6D, 7B,D,F). The higher dose, 1000 µg/kg, shifted daily water intake from the night hours to the evening during the recovery phase (Fig. 6F).

The total time spent drinking was extended in the evenings by the lethal dose to L–E rats (Fig. 7C). In the H/W rats, TCDD elicited only minor alterations in drinking times. The drinking rate was slightly decreased by the 100- (both strains) and 1000-µg/kg doses (H/W) up to day 5. Thereafter, a subtle increase was recorded in L–E rats at 10 µg/kg during the night hours (data not shown).

When the drinking data were broken down by the daily L/D periods, some surprising findings emerged (Suppl. Table 2A,B). Of all experimental groups, total water consumption was lowered only at the highest doses in both strains and only during the dark period. This was due to fewer licks per each drinking session, an effect also recorded in the L–E rats during daytime at 100 µg/kg. However, the L–E rats (but not the H/W rats) compensated for this reduced bout size, mainly by drinking two to three times more frequently than controls in the light hours. Therefore, the proportional water consumption increased in lethally TCDD-treated L–E rats during the illuminated phase and decreased in darkness; their changes in circadian partitioning of these drinking measures were already mostly significant on the day of exposure.

3.5. Effect of TCDD on the sequential patterns of consummatory behavior

In the control rats of both strains, most meals were followed by drinking throughout the day, which contributed to the total food intake by more than 80% (Suppl. Fig. 3A,B). Consecutive meals and

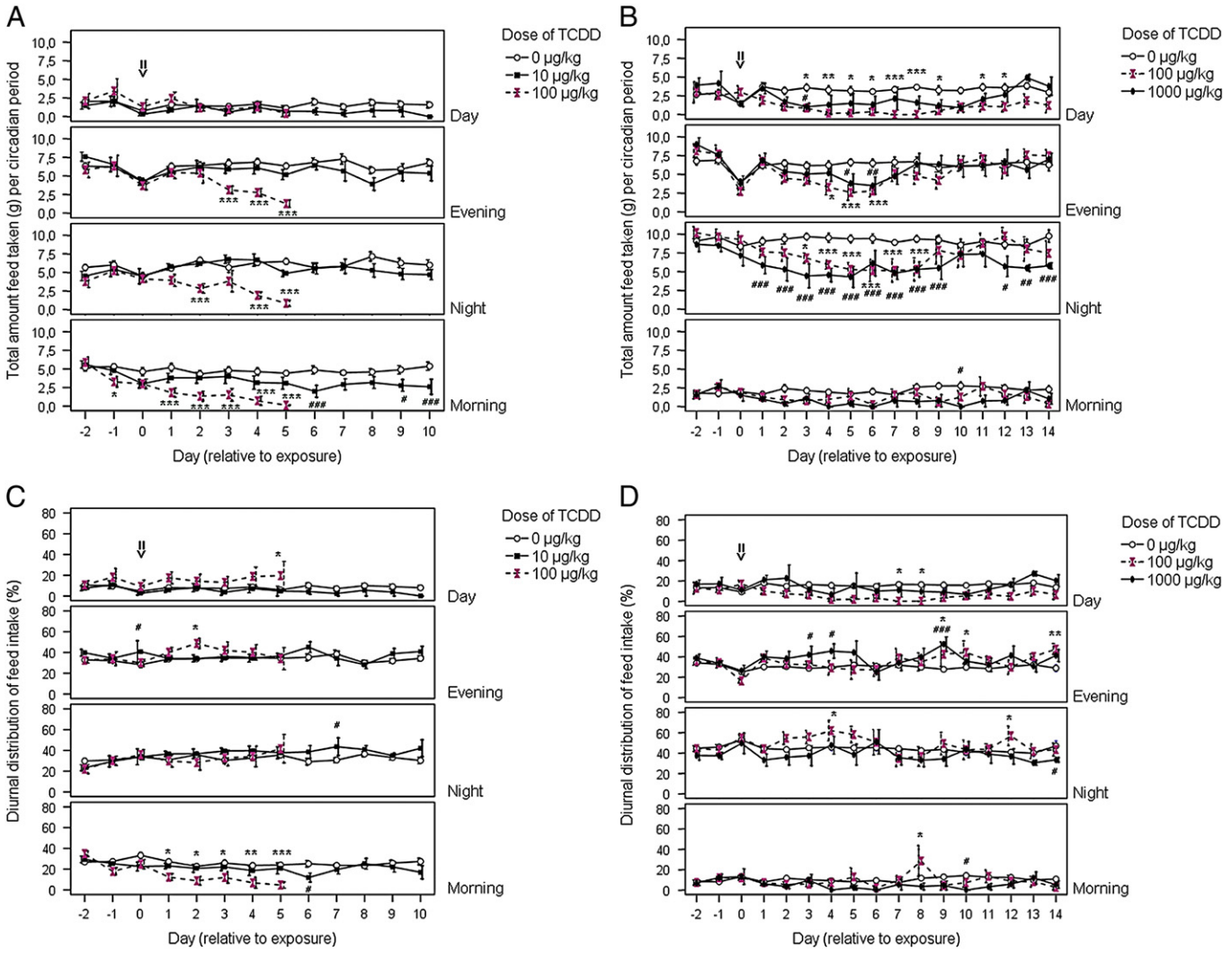

Fig. 3. TCDD exposure decreased feed intake from the feeder both in male L–E rats (A) and in male H/W rats (B). These amounts include spilled feed (shown in Fig. 2C). Panels C and D show the effect of TCDD on diurnal distribution of feeding (distribution of the total amount feed taken from the feeder), (C) in L–E rats and (D) in H/W rats. Group sizes and statistical significances (as assessed by the linear mixed model or by Poisson regression using generalized estimating equations) are as in Fig. 1, treatments are shown on the upper right corner of the panels. The symbols represent mean values and the error bars show the SE. For clarity, only the differences between the controls and TCDD-treated groups are shown. Days were split into four circadian periods: Day (10 a.m.–4.59 p.m.), Evening (5 p.m.–9.59 p.m.), Night (10 p.m.–4.59 a.m.) and Morning (5 a.m.–9.59 a.m.). A downward arrow at the daytime panel depicts the time of TCDD exposure [at noon on day 0].

meals interrupted by a drinking bout appeared seldom, mainly at phases other than daytime.

TCDD exposure tended to decrease the contribution of the most typical sequence, a meal followed by drinking, to the total feed intake. Concomitantly, the proportional intake in consecutive meals and in meals interrupted by drinking increased (Suppl. Fig. 3A,B). The alterations in these behavioral patterns were most discernible during the night hours, especially in H/W rats.

The lethal dose, 100 µg/kg, to L–E rats diminished the meal size independently of the type of behavioral sequence. In the H/W rats, meal size decreased in the meal-to-drinking sequence for the first week postexposure. Thereafter, the meal size increased in sequences other than consecutive meals.

In control rats of both strains, drinking predominantly occurred in two types of behavioral sequence, consecutive drinking bouts and drinking bouts followed by a meal; meals interrupted drinking only rarely. This was true whether the data were expressed relative to daily water consumption (Suppl. Fig. 3C,D) or relative to the number of all drinking events (data not shown).

The TCDD treatment exhibited a clearly bidirectional impact on drinking patterns: at both doses in H/W rats and at the higher dose in L–E rats, the drinking-to-drinking sequence became more prominent and the drinking-to-meal sequence less prominent. There was a similar tendency at the lower dose in L–E rats but it did not attain statistical significance (Suppl. Fig. 3C,D). In L–E rats drinking was also more likely to be interrupted by a meal after TCDD exposure.

3.6. Time lags between meals and drinking bouts

The average lag from a meal to a drinking bout was twice as long in L–E control rats as in H/W controls (46 ± 2 min vs. 24 ± 1 min, respectively), with the difference peaking in the night when the lags were shortest in H/W rats (21 ± 1 min) but longest in L–E rats (mean value 58 ± 4 min). In both L–E groups, TCDD shortened the postprandial time from meal to drinking during the night, whereas in the morning there was a contrasting effect. In H/W rats, a higher dose of 1000 µg/kg prolonged postprandial lags in the morning on days 7–9 and shortened them during daytime (data not shown).

The lags from drinking to meal did not differ between the strains (39 ± 3 min in L–E controls vs. 42 ± 2 min in H/W controls) and were shortest in both strains during the evening and night periods. The

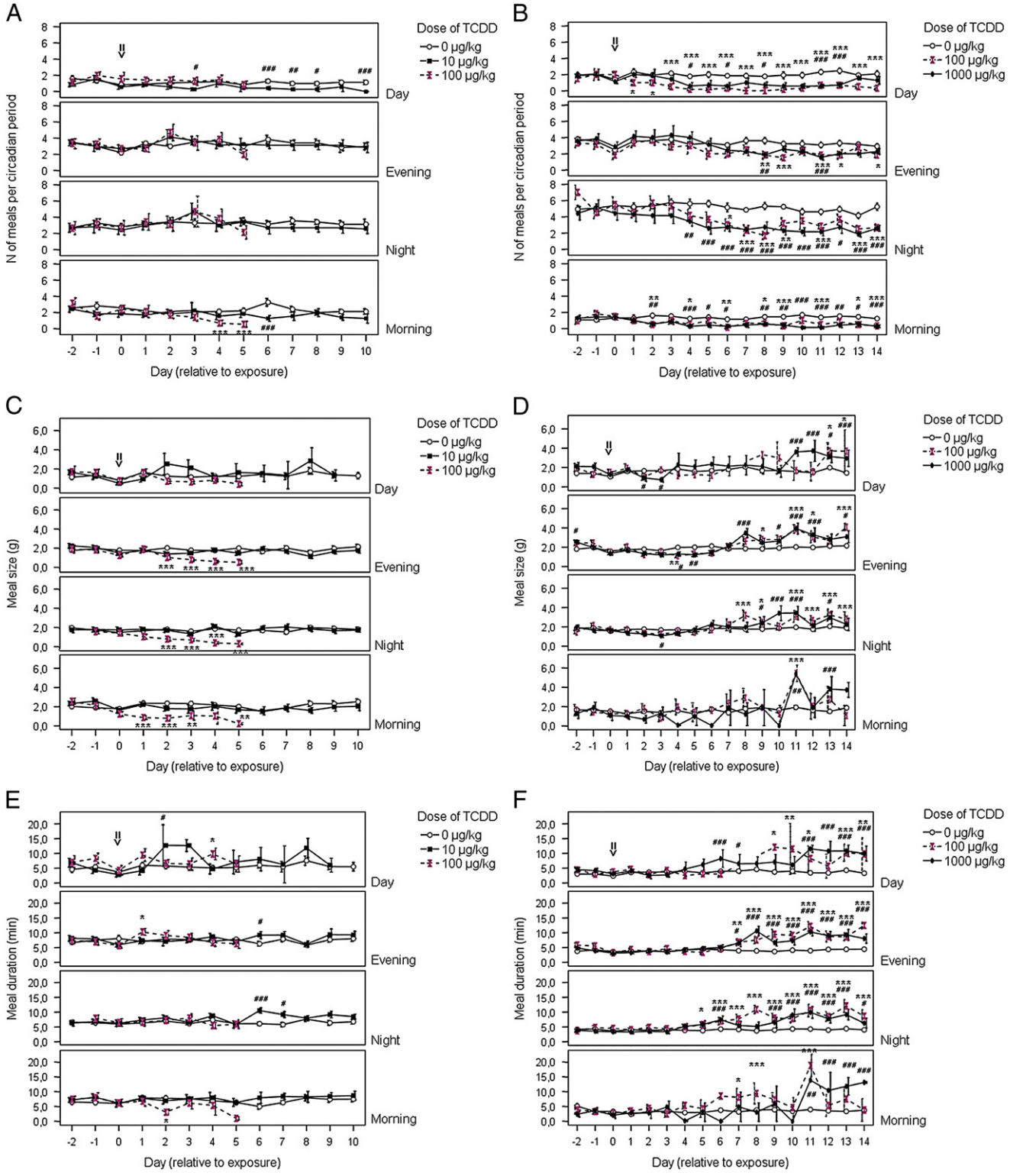

Fig. 4. Effects of TCDD on meal number, meal size (g) and duration (min) at different circadian periods in L–E rats (left panels, A, C and E) and in H/W rats (right panels, B, D and F); the graphs show mean values ± SE. Group sizes are as in Fig. 1 and diurnal periods as well as statistical analyses as in Fig. 3.

100-µg/kg dose to L–E rats shortened this lag during daytime, whereas the 1000-µg/kg dose to H/W rats increased it at night.

4. Discussion

One of the most characteristic overt signs of acute TCDD toxicity in rats is a dramatic BW loss dubbed the wasting syndrome. It is mainly due to reduced feed intake (Kelling et al., 1985; Seefeld et al., 1984a). However, this hypophagia has not previously been subjected to detailed analysis, although the importance of a single meal as a biological unit of eating has been recognized for decades (Brobeck, 1955). In the present study, we utilized two differently TCDD-sensitive rat strains and an automated monitoring system to follow and analyze TCDD-induced alterations in feeding and drinking macro-

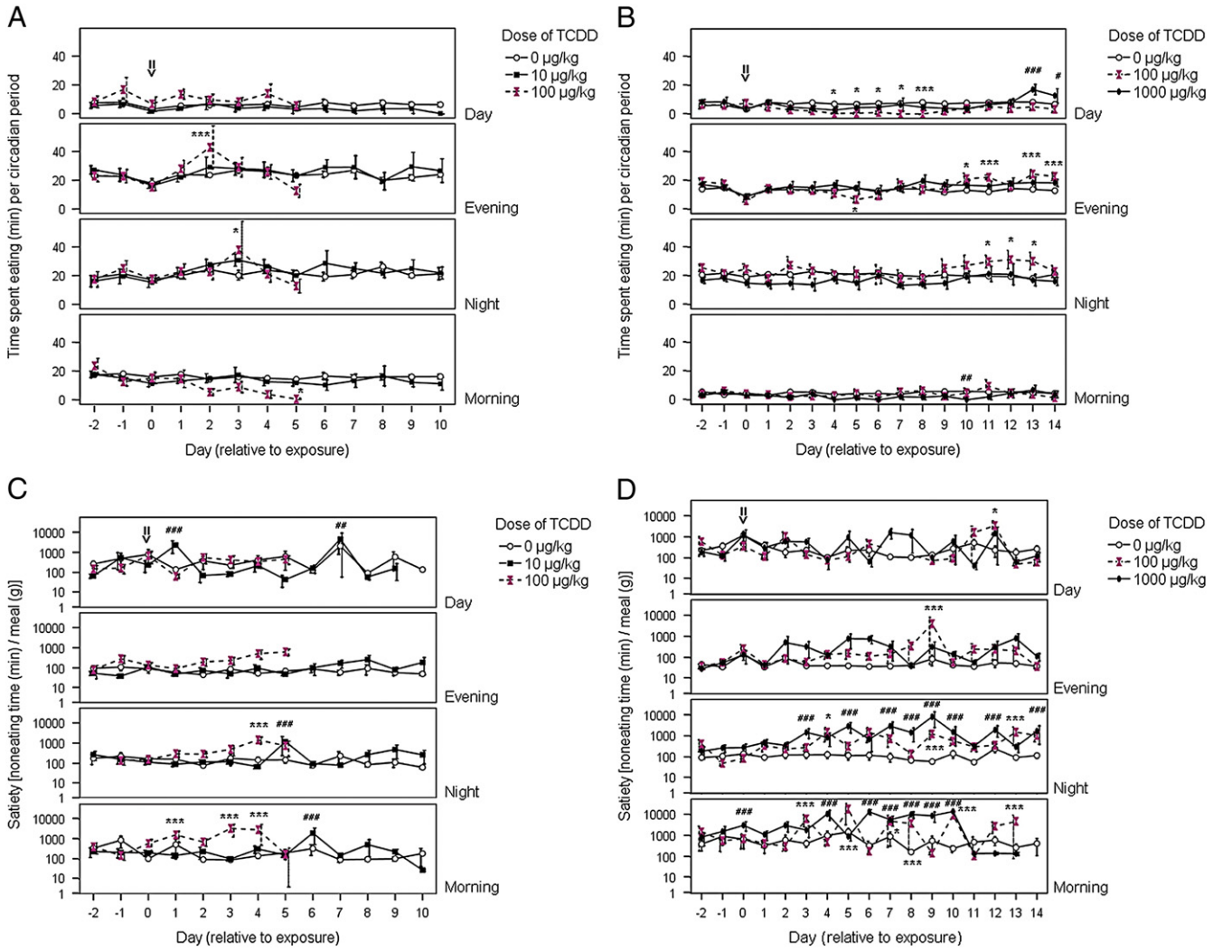

Fig. 5. Panels A (L–E rats) and B (H/W rats) show the total times (min, mean ± SE) spent eating at different times of day. Panels C and D depict calculated satiety values (note the logarithmic scale on the y-axis). Conditions and statistical significances are as in Fig. 1.

and microstructures during the progressive reduction of feed intake, as well as (for TCDD-resistant rats) during its gradual recovery phase. The higher dose (100 µg/kg) employed in L–E rats would ultimately have been 100% lethal; the lower dose (10 µg/kg) is about half the LD_{50} value for this strain (Pohjanvirta et al., 1993). Although notably high for rats, both doses (100 and 1000 µg/kg) used for H/W rats are sublethal to this particular strain with a mutated AHR (Pohjanvirta et al., 1998; Unkila et al., 1994).

Traditionally, feed intake studies have confined analysis to the two chief phases of day: light and dark. Our recent investigations on untreated L–E and H/W rats have unveiled that these two strains exhibit remarkable divergences in their basal feeding behavior, especially with regard to eating at the time of the morning light shift: while this represents a peak feeding activity time in L–E rats, it is the nadir for H/W rats (Lensu et al., 2011a). Therefore, we found it necessary to obtain more detailed—but still manageable—behavioral data from the L/D transitions by dividing the day into four phases. By analyzing these data in combination with the conventional L/D data, it was possible to obtain a much more thorough view of the behavioral changes caused by TCDD than if it were based on either data source alone.

Our in-depth analysis revealed that the TCDD-resistant H/W and TCDD-sensitive L–E rats display clearly distinct alterations in their consummatory behaviors after TCDD exposure, despite the fact that both strains diminish their total feed consumption. While the decline was mainly due to a decreased size of meals in L–E rats, it followed from a reduced frequency of meals in H/W rats. In L–E rats the higher dose principally affected feeding at the morning L/D transition phase, whereas H/W rats chiefly lowered their daytime feed intake. Meal duration remained fairly stable at the early stages of TCDD intoxication in both strains but was substantially elevated in H/W rats during their recovery from the initial decline in feed intake and BW (after the first week postexposure). At that time, they also ate larger meals than the controls. Water consumption was reduced in lethally TCDD-treated L–E rats at night and in the morning, with a shift towards augmented daytime drinking. In the H/W rats, nighttime drinking was strongly suppressed by TCDD as a consequence of the lower number of licks in each bout. Although bout size was also adversely affected by the lethal TCDD dose in L–E rats, they readily compensated for it by drinking more often during the light hours. Overall, the lethal dose shifted drinking activity clearly from darkness dominance towards equal partitioning between the light and dark hours in L–E rats, so that on day 5 both phases contributed by about 50% to total water intake. This phenomenon did not occur in the H/W rats. The lower dose of TCDD tested in L–E rats (10 µg/kg) did not affect total daily feed intake until day 5, although meal size was already diminished during the dark hours on day 2. From day 5 on, this dose of TCDD reduced feed consumption at the morning transition phase and slightly decreased the meal number in daytime.

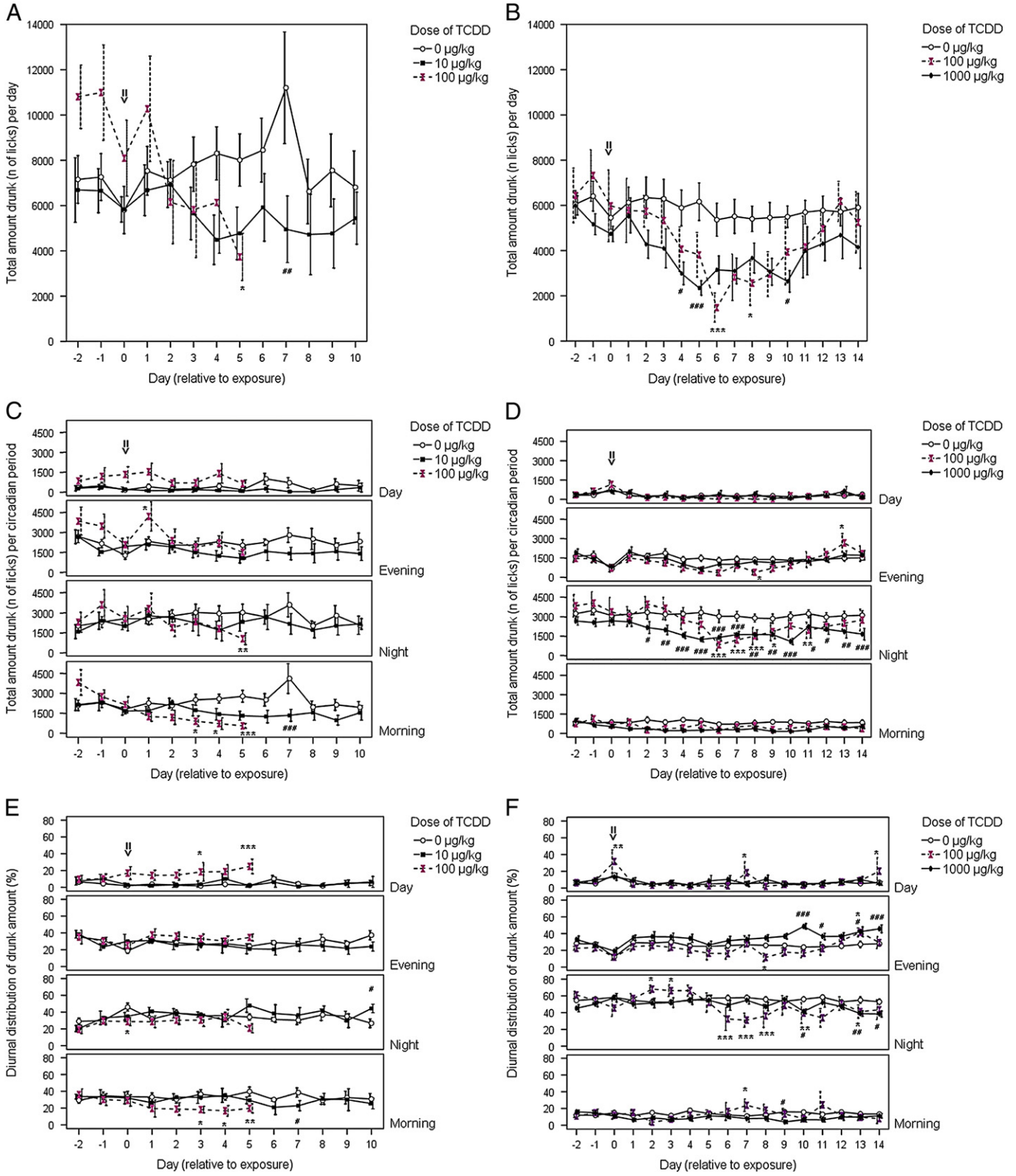

Fig. 6. TCDD-exposed rats tended to drink less than controls. Panel A shows the total daily water consumption (number of licks, mean ± SE) for L–E rats, panel B for H/W rats. The effect of TCDD on drinking was discernible during mornings in L–E rats (C) whereas in H/W rats night-time drinking diminished most (D). In panels E and F, the diurnal distribution of drinking (of the total amount drunk, %) is shown. Conditions, group sizes and statistically significant differences from controls are as in Fig. 4.

The major differences in the responses of the two strains are summarized in Table 1.

A number of physiological, pharmacological, and inflammatory factors and agents affect feed intake by modifying meal size and leaving meal frequency intact, e.g. ghrelin, the central melanocortin system, corticotropin-releasing hormone, benzodiazepine partial agonists, and interleukin-1β (Cottone et al., 2007; Elander et al., 2007; Fekete et al., 2007; Hillebrand et al., 2006). Interestingly, the

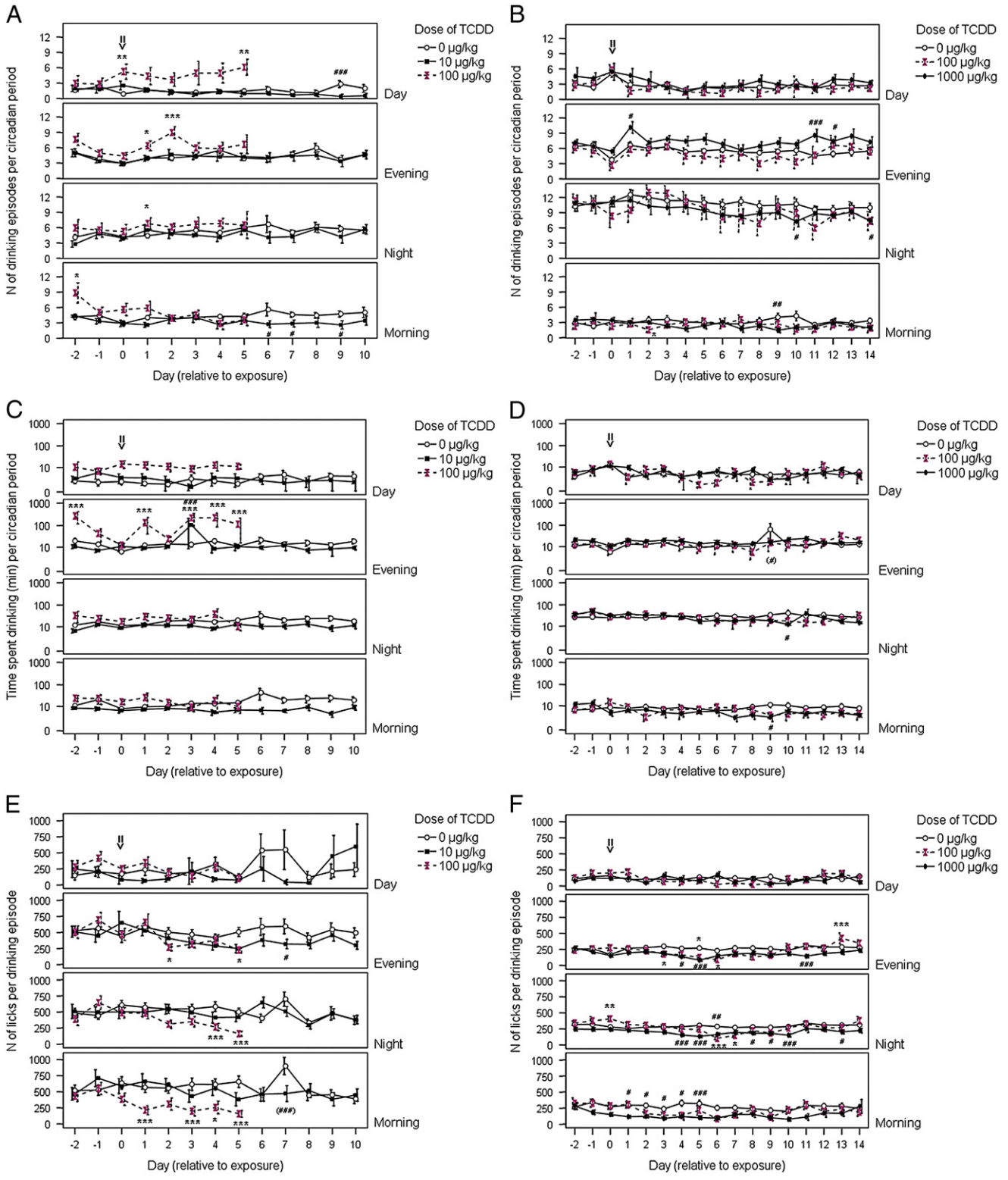

Fig. 7. Mean values (±SE) of total numbers of drinking bouts at different circadian periods in L–E (A) and H/W (B) rats. Panels C and D show, respectively, the total times (min, mean ± SE) spent drinking at different times of day; note the logarithmic scale on the y-axis. Panels E (L–E) and F (H/W) depict mean values for single drinking bout sizes (number of licks, mean ± SE). The control values deviated from the normal level on day 9 for H/W rats (panel D) and on day 7 for L–E rats (panel E), therefore the statistically significant differences are shown in parentheses. Otherwise conditions and statistical significances are as in Fig. 4.

pancreatic peptide glucagon-like peptide 1 decreases meal size, whereas another pancreatic satiety signal, amylin, decreases both meal size and meal number (Reidelberger et al., 2004; van Dijk and Thiele, 1999). The combined pattern is also typical of the anorexia in tumor-bearing rats; however, in that case the reduction of feed intake is at the onset driven by a downward change in meal number, and meal size decreases only later (Meguid et al., 2000b). In mice, lipopolysaccharide reduces meal size, whereas in rats it induces weight loss by lowering meal number (Elander et al., 2007; Geary et al., 2004). These examples imply that meal size and meal number may

Table 1
Major differential impacts of TCDD on consummatory behaviors in L–E and H/W rats.

L–E (10 μg/kg)	L–E (100 μg/kg)	H/W (100 or 1000 μg/kg)
Delayed suppression of feed intake, ~30% reduction from the fifth day on	Irreversible, severe suppression of feeding	Reversible, 50% reduction of feed intake
Feed intake (g) most affected in morning hours; not affected in daytime	Feed intake (g) most affected in morning hours; not affected in daytime	Feed intake (g) most affected in daytime and during night
Initially meal size reduced (in dark hours)	Meal size the main variable affected	Meal frequency the main variable affected
Minor effect on meal duration	Minor effect on meal duration	Meal duration substantially increased in the recovery phase
Drinking slightly diminished throughout the day, diurnal distribution unaltered	Drinking diminished in the morning and during night with a shift towards enhanced daytime drinking	Drinking markedly lowered in the night hours due to lowered bout size
Drinking frequency unaltered	Drinking frequency increased in light hours (from day 0 on)	Drinking frequency unaltered

have distinct regulatory systems. In support of this interpretation, two key hypothalamic centers of energy balance regulation, the ventromedial hypothalamic nucleus (VMN) and lateral hypothalamic area (LHA), influence meal number and meal size, respectively (Meguid et al., 2000a). Dopamine and serotonin (5-HT) are neurotransmitters playing important roles also in regulation of food intake. 5-HT is able to regulate dopamine release (Giannakopoulos et al., 1998; West and Galloway, 1996) in addition to its own anorectic activity (Laviano et al., 2009), and they have shown to interactively affect meal number in VMN and meal size in LHA (Meguid et al., 2000a). While TCDD in the present study diminished feed intake in both L–E and H/W rats, it did so by affecting meal size in L–E rats but meal number in H/W rats. Since the higher TCDD doses employed here lead to a fatal wasting in L–E but to a largely reversible hypophagia in H/W rats, the microstructural feeding analysis outcome suggests that the feeding regulatory pathway critically derailed in L–E rats may remain untouched in H/W rats in which TCDD instead may modify another, much less detrimental pathway. This second pathway may require such high doses that it will never become discernible in strains sensitive to TCDD lethality. For verification of this hypothesis, further studies in TCDD-sensitive rat strains are needed. The increase in meal size recorded in H/W rats during their recovery phase is consistent with a response to elevated levels of the main peripheral hunger signal, ghrelin (Cottone et al., 2007).

As reported earlier (Pohjanvirta et al., 1987), the feeding response of H/W rats to TCDD exposure was clearly biphasic. The initial suppression of feed intake faded by 6–7 d and was followed by a swift recovery to nearly control intake levels. This pattern is in agreement with our previous results of messenger RNA (mRNA) levels in the hypothalamus, which showed that exposure to 50 μg/kg TCDD diminished expression of orexigenic factors in L–E rats after 6 h but only after 24 h in H/W rats; the decrease turned into an increase after 96 h (Lindén et al., 2005). As stated in the Introduction, despite numerous studies the biochemical basis of the wasting syndrome has remained elusive. The role of the central nervous system is still largely an open question (Lindén et al., 2010), and the reported changes in intermediary metabolism are frequently inconsistent or even contradictory (Birnbaum and Tuomisto, 2000; Lindén et al., 2010; Pohjanvirta and Tuomisto, 1994).

A change in rats' circadian feeding rhythms after TCDD exposure was reported earlier (Christian et al., 1986). It also emerged in our previous study in L–E rats exposed to a low-lethal dose of TCDD (20 μg/kg) as a result of decline in feeding, mainly during the dark phase (Pohjanvirta et al., 1988). In the present study, the high dose of 100 μg/kg TCDD to L–E rats increased daytime feed and water intakes relative to their total daily consumptions. Both feeding and drinking diminished at the morning L/D transition phase, which represents the peak feeding phase in L–E (but not H/W) rats (Lensu et al., 2011a). As a result, the proportional daytime intakes increased. However, as described above, the lethal dose of TCDD also increased the frequency of drinking bouts during the light hours, and it is notable that this change emerged already on the day of exposure. A more prominent shift in circadian timing was recorded in drinking than in feeding, suggesting that there may be some selectivity in the impacts of TCDD on water and feed intake rhythms in L–E rats. The sublethal dose of 10 μg/kg was surprisingly innocuous in this respect, considering that it caused an almost 10% weight loss by day 11. Yet, the morning transition phase again appeared to be the most susceptible time of day to TCDD in this strain.

Our previous studies also disclosed that from about 2 wk after exposure to a large dose of TCDD (1000 or 3000 μg/kg), H/W rats exhibit a tendency towards elevated feed intake during the light hours (measured for 5-h periods) (Pohjanvirta and Tuomisto, 1990a, 1990b). In the present study, this temporal trend was not evident. However, the observation period only spanned the first 2 wk, while several days before termination the amount eaten during daytime showed an upward trend at 1000 μg/kg of TCDD, with the nighttime consumption starting to decrease. Thus, this change in circadian feeding rhythms appears to be a delayed phenomenon, emerging at a time when H/W rats have resumed eating at levels close to their preexposure intake values.

The exceptionally wide sensitivity difference between L–E and H/W rats to TCDD toxicity is mainly based on structural modifications in the H/W rat AHR (Pohjanvirta, 2009; Pohjanvirta et al., 1998; Tuomisto et al., 1999b). The AHR has been implicated in the regulation of diurnal rhythms (Mukai et al., 2008; Mukai and Tischkau, 2007), in addition to its well-established role in xenobiotic metabolism and in several other physiological functions [reviewed in Lindén et al. (2010); Rannug and Fritsche (2006); Shimba and Watabe (2009)]. For example, tryptophan photoproducts seem to modulate the regulation of light-dependent circadian rhythms via an AHR-dependent pathway (Mukai and Tischkau, 2007). Tryptophan metabolites belong to naturally occurring and endogenous AHR agonists. Hence it is possible that a 5-HT-regulated pathway is involved in feeding micro- and macrostructures with TCDD interfering with this regulation, because the serotonergic system knowingly participates in regulation of e.g. feeding, sleep–wake and circadian rhythmicity (Meguid et al., 2000a; Laviano et al., 2009; Rosenwasser, 2009). The serotonergic responses in these rat strains have been shown to differ following TCDD. In L–E rats brain 5-HT turnover increased together with elevated levels of plasma free tryptophan in a dose-responsive manner whereas in H/W rats this did not occur (Unkila et al., 1994).

Circadian rhythmicity involves interplay of many neurotransmitters known to exhibit circadian and sensory-stimulated fluctuation (Rosenwasser, 2009). Although no studies have so far demonstrated the circadian expression of the AHR in these rat strains or the effects of TCDD on it, AHR protein levels are known to oscillate in several tissues of female Sprague–Dawley rats on a diurnal basis, showing a peak during the daytime (Richardson et al., 1998). Activated AHR represses the rhythmic expression of period 1(Per1) in mouse liver, possibly through disrupting the activity of circadian locomotor cycles kaput-brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (CLOCK-BMAL1) (Xu et al., 2010), while Per1 as well as period 2 (Per2) appear to mediate the diurnal fluctuation in responsiveness to CYP1A1 induction in mouse liver and mammary

gland (Qu et al., 2010). A previous study from this laboratory recorded no changes in Per2 mRNA expression in the hypothalamus following TCDD administration. Unfortunately, the presence of Per1 mRNA was not determined. However, the basal levels of Per2, AHR, and ARNT mRNAs were 2–3-fold higher in L–E than H/W rats (Korkalainen et al., 2005). These may play a role in the differences in circadian feeding and drinking responses between these rat strains in the basal state (Lensu et al., 2011a) and following TCDD exposure.

For the definition of a meal, a minimum threshold value for meal size was set in some studies (e.g. Geary et al., 2004; Glendinning and Smith, 1994). In the present study all bouts were included, and it appeared that in both strains TCDD increased the number of smallest meals, which is associated with an increase in the mean eating rate (g/min). It has also been demonstrated that the definition of a meal or a drinking bout influences the results in analyses of consummatory behavior (Hulsey et al., 1998; Johnson et al., 2010; Zorrilla et al., 2005). In both strains, sequential patterns of feeding and drinking were altered, and although these patterns are sensitive to meal definitions (e.g. whether or not immediate postprandial drinking is included in a meal), TCDD exposure diminished the typical rodent behavior of having a drinking episode right after a meal, or in the opposite case, a meal after drinking. In rats, most drinking is temporally associated with feeding and this seems to be regulatory (Fitzsimons and Le Magnen, 1969; Zorrilla et al., 2005). It appears that TCDD interacts with these regulatory pathways; in addition to proportional intakes in distinct sequences, the lags between them were modulated by TCDD exposure. Nevertheless, since this is the first detailed analysis of TCDD-induced impacts on consummatory behaviors, we addressed feeding and drinking separately. To gain further insight into structures of episodes or food-associated drinking, a more detailed analysis should be done.

A consistent feature of TCDD-induced hypophagia is feed spillage, which increases dose-dependently and which was first described almost three decades ago (Seefeld et al., 1984a). It was also found in the present study in both strains; however, the magnitude was much more pronounced in lethally dosed L–E rats, in which it amounted to over 30% of the total daily intake by day 5. Our microstructural feeding analysis did not take the spilled feed into account, because it was impossible to estimate afterwards the exact time or the episode in which each pellet was wasted. On the other hand, although the pellets spilled by a given rat were not consumed, they still indicated its motivation to eat or forage. The reason for spilling is unknown. Spilling behavior may reflect ambivalence in feeding behavior: the rat is motivated to eat, but for an unknown reason feeding is discontinued and the feed pellet dropped. The increased frequency of consecutive meals is in support of this. However, there is also another plausible explanation, in that it may be a reflection of hoarding behavior. A well-established behavioral response in rats (and many other animals including hamsters and birds) to feed restriction and other manipulations causing BW loss is to begin hoarding food. This behavior can be seen as an alternative to fat accumulation as a means to store dietary energy (Keen-Rhinehart et al., 2010). In our previous studies, we often observed lethally TCDD-treated L–E rats, maintained on a regular large-pellet (several grams) diet, to garner stacks of pellets in a corner of their cage (unpublished data), supporting the view that TCDD exposure triggers hoarding behavior. The reduced eating rate in the case of large meals detected here is also compatible with the idea of the rats carrying feed pellets in an attempt to hoard them. Interestingly, hoarding is a quantifiable measure that negatively correlates with changes in BW, is enhanced by the feeding-stimulating factors ghrelin and neuropeptide Y, and is inhibited by the fat-derived satiety factor leptin (Keen-Rhinehart et al., 2010). It has been proposed to represent the externally detectable indicator of BW set-point (Fantino and Cabanac, 1980). Since a major hypothesis of the wasting syndrome interprets it as a consequence of lowered BW set-point (Seefeld et al., 1984b), further studies on the relationship between hoarding and TCDD exposure would be highly warranted.

5. Conclusions

This study is the first to provide a detailed analysis of feeding and drinking behaviors of TCDD-treated rats. We found clear indications that different regulatory pathways may underlie the responses recorded in TCDD-sensitive vs. TCDD-resistant rats and that the AHR may play a modulating role in the circadian rhythms of feeding and drinking. Since the wasting syndrome is a model of fatally derailed regulation of BW and energy homeostasis, resolving its biochemical pathogenesis will further our understanding of these vitally important but intricate systems.

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The authors declare that the study sponsors played no role in study design; in the collection, analysis, and interpretation of the data; in the writing of the report; and in the decision to submit the paper for publication.

Disclosure statement

The authors had no actual or potential conflict of interest, including any financial, personal, or other relationships with other people or organizations within three (3) y of beginning the work submitted that could have inappropriately influenced (biased) this work.

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