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# Exposure to endocrine disrupting chemicals perturbs lipid metabolism and circadian rhythms

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ABSTRACT

A growing body of evidence indicates that exposure to environmental chemicals can contribute to the etiology of obesity by inappropriately stimulating adipogenesis as well as perturbing lipid metabolism and energy balance. One potential mechanism by which chemical exposure can influence lipid metabolism is through disturbance of circadian rhythms, endogenously-driven cycles of roughly 24hr in length that coordinate biochemical, physiological, and behavioral processes in all organisms. Here we show for the first time that exposure to endocrine disrupting compounds (EDCs), including the pesticide tributyltin, two commercial flame retardants, and a UV-filter chemical found in sunscreens, can perturb both circadian clocks and lipid metabolism in vertebrates. Exposure of developing zebrafish to EDCs affects core clock activity and leads to a remarkable increase in lipid accumulation that is reminiscent of the effects observed for longdaysin, a known disruptor of circadian rhythms. Our data reveal a novel obesogenic mechanism of action for environmental chemicals, an observation which warrants further research. Because circadian clocks regulate a wide variety of physiological processes, identification of environmental chemicals capable of perturbing these systems may provide important insights into the development of environmentally-induced metabolic disease.


This study examined the effects of exposure to EDCs on lipid metabolism and circadian rhythms. Zebrafish (*Danio rerio*) are an appropriate vertebrate model for this purpose, as their digestive organs and processes of lipid synthesis and transport are similar to those of humans (Schlegel and Gut, 2015). Isoforms of peroxisome proliferator activated receptors (PPARs), the primary lipid sensors in vertebrates, are highly conserved between humans and zebrafish (Schlegel and Gut, 2015). In addition, most of the zebrafish clock genes show not only a high degree of sequence similarity to their human homologs, but similar function as well (Pando and Sassone-Corsi, 2002). As in mammals, zebrafish genes *clock* and *bmal1* are the primary circadian oscillators, initiating transcription of *period* and *cryptochrome* genes (Pando and Sassone-Corsi, 2002). Resulting dimers of Period and Cryptochrome inhibit the formation of Clock: Bmal1 complexes to create a negative feedback loop repressing their own transcription. Expression of Period1 can also be suppressed by Ppar$\gamma$, the central regulator of adipogenesis (Kawai et al., 2010).

We used a transgenic zebrafish line Tg (4xEbox:Luc) expressing luciferase driven by four E-boxes, representing binding sites for Clock/Bmal (Weger et al., 2013). From 6 days post fertilization (dpf) on, zebrafish larvae were fed a standard diet consisting of 4 ml of live *Tetrahymena* suspension twice

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daily plus 6 mg powdered fish baby food (Sera micron) containing about 0.5 mg lipids. These larvae were compared with larvae fed a hypercaloric diet (HCD), in which one feeding per day was replaced by 4 mL boiled chicken egg yolk suspension containing about 50 times higher lipids than the standard diet. From 9 to 14 dpf, we added non-toxic concentrations of endocrine disrupting compounds (EDCs) to their medium, simulating environmental conditions with no observable effects on growth and development (see figure legends for experimental details). On day 15 we monitored core clock activity as described previously (Weger et al., 2013). We also stained the larvae with Nile Red to visualize intracellular lipid droplets, particularly triglycerides (Jones et al., 2008), and quantified signal density. In order to determine if the effects of EDCs on lipid metabolism could be mediated by changes in circadian rhythms, we selected EDCs known to affect PPAR signaling. These included tributyltin (TBT), an organotin compound used as an antifungal agent, a PVC stabilizer, and protective agent for wood, which is known to modulate RXR-PPAR dimerization and promote adipogenesis (Grun and Blumberg, 2009). Another EDC was tetrabrominated bisphenol A (TBBPA), a widely-used brominated flame retardant and plasticizer used in coatings, adhesives, paper, and children's clothing, which has been shown to disrupt both thyroid hormone receptor activity and Pparγ signaling (Riu et al., 2011). We also tested tris (1,3-dichloroisopropyl) phosphate (TDCIPP), a chlorinated organophosphate used for polyurethane foams that is being increasingly used as a flame retardant to replace polybrominated diphenyl ethers. TDCIPP is a known agonist of estrogen receptor α and increases mRNA expression of Pparα-centered gene networks (Liu et al., 2013). Finally, we also selected benzophenone 3 (BP-3), a candidate obesogen that is not known to affect PPAR signaling. BP-3 is an organic compound used in sunscreens that absorbs UVB and UVA radiation, which has been shown to have estrogenic properties (Krause et al., 2012).

All EDCs tested in zebrafish demonstrated obesogenic effects, as visualized and quantified by fluorescent lipid staining in the trunk area between the gall bladder and proximal intestine, *i.e.*, the pancreatic region where the greatest accumulation of lipids occurs under control conditions (Fig. 1a) and where adipocytes will first form (Flynn et al., 2009). As expected, feeding larval zebrafish with a hypercaloric diet (HCD) significantly increased the fluorescent lipid signal relative to the controls by a factor of 1.6 (Fig. 1b). Exposure of larval zebrafish to EDCs under a standard diet, however, resulted in even higher lipid accumulation. Exposure to the pesticide TBT significantly increased lipid signal density by a factor of 3.6 relative to controls, while BP-3, TBBPA, and TDCIPP also significantly induced lipid accumulation to levels of 2.7- to 3.4-fold (Fig. 1c). Though obesogenic effects of TBT, TBBPA and TDCIPP through Pparγ signaling has been shown before (Grun and Blumberg, 2009; Riu et al., 2011; Liu et al., 2013), this is to our knowledge the first report of stimulation of lipid accumulation by BP-3. The lipid accumulation elicited by EDCs was more restricted to the defined pancreatic region compared to HCD, where increased lipid accumulation was also visible in the liver, heart, and gills (Fig. 1a). Polyunsaturated fatty acids, such as those present in egg yolk, are known to bind and activate Pparγ (Bordoni et al., 2006), thereby stimulating adipogenesis. A dietary overload of fatty acids can stimulate all cells to form and sequester neutral lipids within droplets (Greenberg et al., 2011), an effect that was observed in the HCD larvae.

All EDCs tested also affected core clock activity (Fig. 2). Using the luciferase measurements obtained from monitoring transgenic Tg (4xEbox:Luc) zebrafish during a 24-hr period, we determined third degree polynomial regression lines of high robustness (Fig. 2a-b, $R^2 > 0.8$) by which the ability to sustain daily biphasic oscillations with an exact period length has been defined (Hogenesch and Herzog, 2011). Under a control light–dark cycle, transgenic larvae displayed the characteristic oscillations of reporter activity (Fig. 2a), while larvae treated with TBT showed reduced amplitude of oscillations and a prolongation of the period between maximum and minimum activity (Fig. 2a), a period that was prolonged even further following treatment with TDCIPP (Fig. 2b). Larvae exposed to TBBPA or BP-3 displayed a loss of characteristic oscillations with patterns more jagged than those of controls (Fig. 2b). Interestingly, HCD larvae also showed a distinct change in core clock activity patterns with dampened waves and multiple peaks within 24 hr (Fig. 2b). Because Pparγ down-regulates the clock gene *period1* (Kawai and Rosen, 2010) that in turn represses formation of the Clock/Bmal complex, it is possible that clock activity is modulated when the presence of excess fatty acids activates Pparγ signaling.

Given our results indicating that exposure to obesogenic EDCs perturbs clock activity, we were interested to examine the effect on lipid metabolism of chemicals shown previously to alter clock activity. We tested longdaysin and lithium chloride, chemicals known to have different effects on the period of biological clocks in mammals and zebrafish. While longdaysin, a purine derivative, impedes Period1 degradation and slows the circadian clock in a concentration-dependent manner, lithium chloride, widely used in treatment of bipolar disorders, enhances Period2 oscillation amplitude and lengthens the circadian period (Weger et al., 2013). In our experiments, exposure of zebrafish larvae to longdaysin appreciably prolonged the period between maximum and minimum reporter expression, while lithium chloride increased the amplitude of oscillations and shifted but shortened the period between maximum and minimum activity (Fig. 2a). Both chemicals also altered lipid accumulation, although in opposing manners, with longdaysin significantly increasing lipid accumulation 4.3-fold relative to controls, and lithium chloride reducing lipid accumulation compared to controls (Fig. 1d). Both chemicals are known to modulate clock regulatory kinases; lithium blocks Gsk-3β activity regulating lipid accumulation (Freland and Beaulieu, 2012) while longdaysin targets Erk2, a kinase involved in basal lipid droplet formation (Andersson et al., 2006). Thus, both compounds may affect pathways that connect lipid metabolism and circadian rhythms.

We also exposed larvae to two agents known for reducing lipid levels: resveratrol, a natural phenol which decreases total triglyceride content by inhibiting fatty acid synthase (Carten and Farber, 2009), and nicotinic acid, a widely-prescribed drug for lowering plasma triglycerides that inhibits fat-mobilizing lipolysis in adipose tissue (Carlson, 2005). Exposure to either compound modestly decreased lipid accumulation (Fig. 1d) but only site-specifically (Fig. 1a); in each group only one out of 10 larvae showed pancreatic lipid staining compared to controls

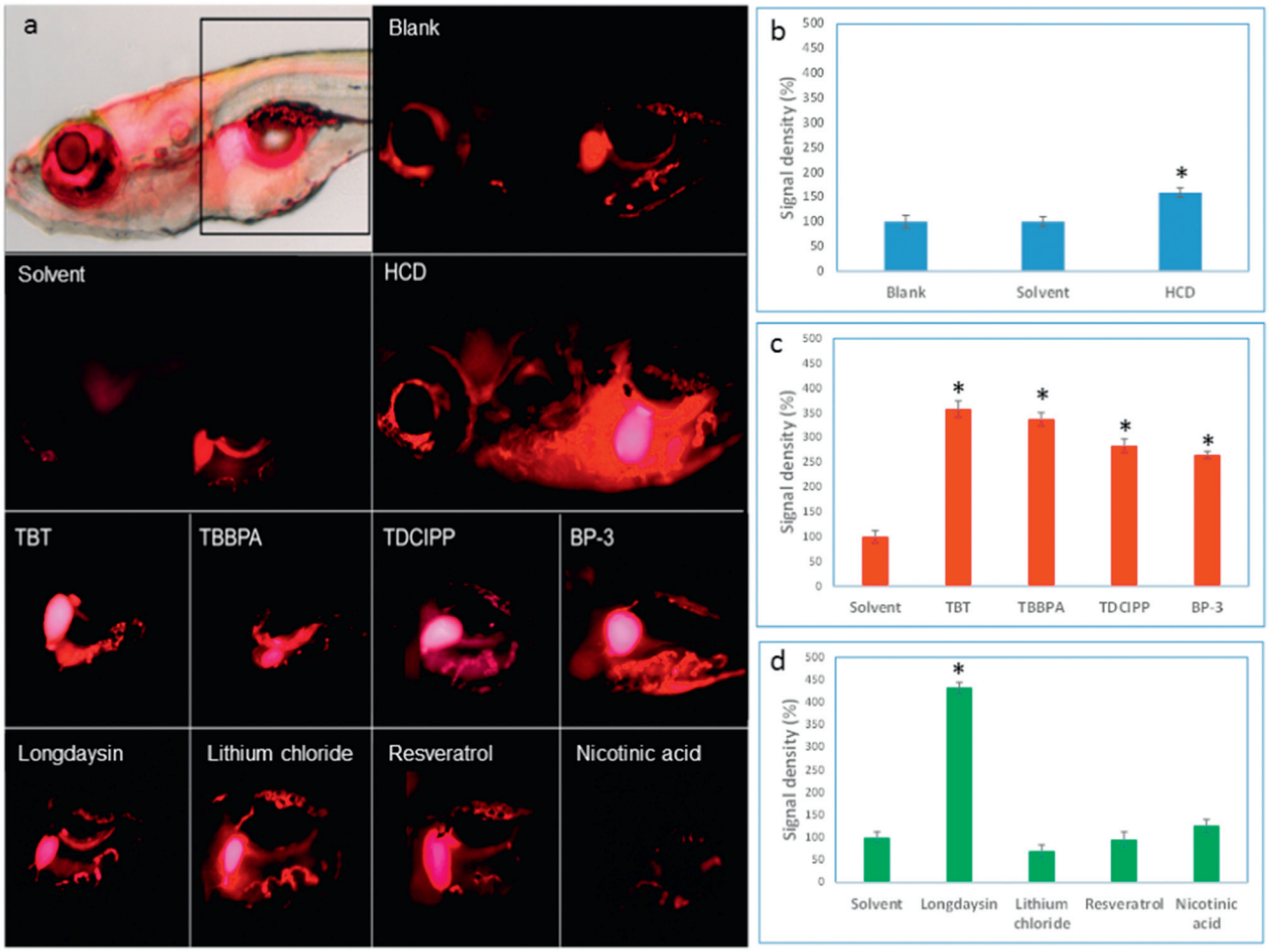


**Fig. 1 – Endocrine disrupting chemicals affect lipid accumulation in zebrafish larvae. (a) Lipid accumulation visualized with Nile Red staining. Method: After 5 days of exposure to chemicals or high caloric diet (HCD) under a 14-hr light/10-hr dark cycle at 26°C, zebrafish larvae (14 dpf) were incubated with Nile Red (50 ng/mL) for 30 min. Larvae were subsequently mounted and orientated in methylcellulose containing tricaine methane sulfonate (MS222). Head and trunk of larvae were imaged by a confocal microscope (Leica DM IL LED, Germany) with a HI PLAN 20×/0.3 objective connected to a digital camera detecting red emission with 543 nm excitation and 560 nm longpass emission filters. TBT (0.001 μmol/L), TBBPA (0.5 μmol/L), TDCIPP (0.5 μmol/L), BP-3 (0.5 μmol/L), long days in (0.9 μmol/L), resveratrol (50 μmol/L), and nicotinic acid (1000 μmol/L) were dissolved in 0.01% solvent (dimethylsulfoxide, solvent) in embryo medium (60 μmol/L $NaHCO_3$, 10 μmol/L $KHCO_3$, 68 μmol/L $CaCl_2{\cdot}7H_2O$, and 37 μmol/L $MgSO_4{\cdot}7H_2O$). Lithium chloride and HCD were dissolved in embryo medium without DMSO (blank). Lipid accumulation quantified with ImageJ after exposure to (b) blank, solvent control (DMSO) and HCD larvae; (c) endocrine disruptors (d) mammalian clock gene disruptors. Method: Images of Nile Red-stained zebrafish larvae were analyzed using ImageJ (National Institute of Health, USA). A fixed area (see box, Fig. 1a) covering the internal organs and trunk muscles was analyzed for integrated signal density. All values were normalized to size to avoid biasing effects of larval size differences. Size was determined by measuring the distance from the most rostral point of the eye to the gall bladder and then on to the most caudal point of the swim bladder, points chosen because they are readily identifiable. Data are shown as percentage change compared with the solvent control (mean of 10 larvae ± SE). ($^*p < 0.05$, one-way ANOVA followed by a posthoc test).**

which showed pancreatic staining in nine out of 10 larvae. Resveratrol did not affect core clock activity, while nicotinic acid markedly reduced maximum reporter activity (Fig. 2a).

Taken together, our data indicate that both lipid metabolism and circadian clock can be manipulated by exposure to EDCs. Comparison of the timing and values of maximum and minimum reporter expression and subsequent cluster analysis illustrated that degrees of lipid accumulation may be inferred from alterations in core clock activity (Fig. 2c). Obesogenic chemicals are distinguished from lipid-lowering chemicals by their effects on clock activity. Larvae exposed to TBBPA and BP-3, as well as HCD, showed a reduction in circadian clock robustness while the distinct biphasic pattern disappeared, allowing for the speculation that TBBPA and BP-3 might affect

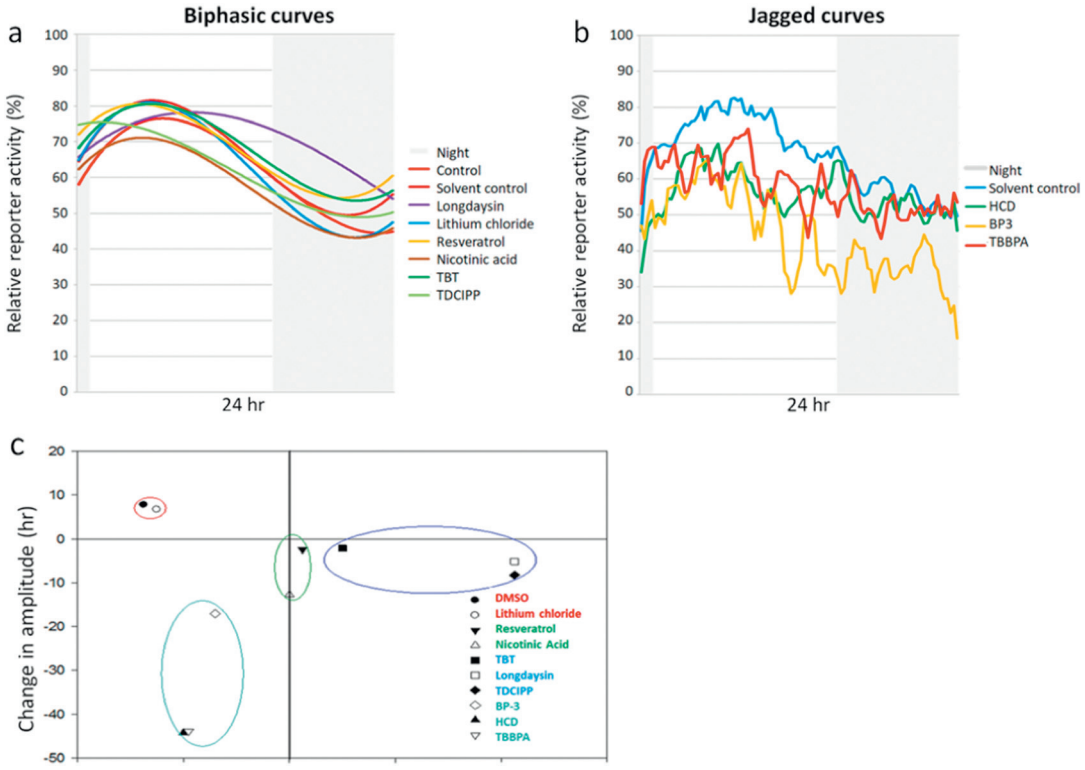


**Fig. 2 – Endocrine disrupting chemicals affect core clock activity. After 5 days of chemical exposure (see legend Fig. 1 for method), oscillating core clock activity was analyzed in 14 dpf larvae under constant dark conditions for 24-hr at 26°C. Individual larvae were transferred to the wells of an opaque 96-well plate and 0.5 mmol/L luciferin in embryo medium (pH 7) was added as described previously (Weger et al., 2013) with measurements performed under red light conditions. Larvae were pre-incubated for 5 hr and luciferase activity was monitored over a 24-hr period in a luminescence plate reader (VARIOSKAN, Thermo Fisher Scientific Inc., Waltham, MA, USA) that measured each well for 5000 msec every 15 min (96 readings/well). Moving averages over four values were calculated. The mean values of at least eight randomly selected larvae were plotted by setting the maximum value to 100% (relative reporter activity). Trend lines were calculated using a cubical best fit in MATLAB (version R2014b, The MathWorks, Natrick, Massachusetts, USA). (a) Under control conditions, the trend line of core clock activity shows a characteristic biphasic pattern with a maximum activity during the light period. Exposure to longdaysin, lithium chloride, resveratrol, nicotinic acid, TBT, and TDCIPP did not affect this pattern ($R^2 > 0.8$) but altered the amplitude and the period between maximum and minimum reporter activity. (b) Jagged trend lines after exposure to HCD, BP-3, and TBBPA showing reduced robustness of circadian rhythmicity (trend line $R^2 < 0.7$). (c) The temporal shift of minimum reporter activity plotted against the fold change in amplitude resulted in a clustering of chemicals.**

some of the same pathways as the hypercaloric diet. On the other hand, exposure to the other two obesogenic EDCs (TBT and TDCIPP) resulted in maintenance of the biphasic pattern while affecting amplitude and periodicity in a manner similar to that of clock gene modulator longdaysin (Fig. 2c).

We provide here a first indication that exposure to endocrine disrupting chemicals can affect both lipid metabolism and circadian rhythms. Though further concentrations should be tested to confirm our data, as well as other EDCs, our results are important due to their potential implications for human health. The EDCs tested in this study have been detected in humans, particularly in placenta and in developing children (WHO, 2013). Early exposure to EDCs has been linked to increased incidence of obesity, diabetes, cancer, and reproductive and neurological dysfunction (WHO, 2013), and is likely to contribute substantially to the already high costs of treating these diseases (Trasande et al., 2015). Confirmation of EDCs as disruptors of circadian rhythms emphasizes the need to control these compounds in the environment. Importantly, a better understanding of the link between regulation of circadian cycles and regulation of lipid metabolism could pave the way to new therapeutic approaches for alleviating the global epidemic of obesity in humans.

## Ethics statement

This study was carried out in strict accordance with the recommendations in care and use of laboratory animals of the directive of the Dutch Parliament. The protocol was approved by the Committee on the Ethics of Animal Experiments of the VU University Amsterdam (DEC IVM13-01).

## Conflict of interest

There are no competing financial interests in relation to this work.

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## REFERENCES

Andersson, L., Bostrom, P., Ericson, J., Rutberg, M., Magnusson, B., Marchesan, D., et al., 2006. PLD1 and ERK2 regulate cytosolic lipid droplet formation. J. Cell Sci. 11, 2246–2257.

Bordoni, A., Di, N.M., Danesi, F., Biagi, P.L., 2006. Polyunsaturated fatty acids: from diet to binding to ppars and other nuclear receptors. Genes Nutr. 1/2, 95–106.

Carlson, L.A., 2005. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. J. Intern. Med. 258/2, 94–114.

Carten, J.D., Farber, S.A., 2009. A new model system swims into focus: using the zebrafish to visualize intestinal metabolism *in vivo*. Clin. Lipidol. 4/4, 501–515.

Flynn 3rd, E.J., Trent, C.M., Rawls, J.F., 2009. Ontogeny and nutritional control of adipogenesis in zebrafish (*Danio rerio*). J. Lipid Res. 50/8, 1641–1652.

Freland, L., Beaulieu, J.M., 2012. Inhibition of GSK3 by lithium, from single molecules to signaling networks. Front. Mol. Neurosci. 5, 14.

Greenberg, A.S., Coleman, R.A., Kraemer, F.B., McManaman, J.L., Obin, M.S., Puri, V., et al., 2011. The role of lipid droplets in metabolic disease in rodents and humans. J. Clin. Invest. 121 (/6), 2102–2110.

Grun, F., Blumberg, B., 2009. Endocrine disrupters as obesogens. Mol. Cell. Endocrinol. 304/1–2, 19–29.

Hogenesch, J.B., Herzog, E.D., 2011. Intracellular and intercellular processes determine robustness of the circadian clock. FEBS Lett. 585/10, 1427–1434.

Jones, K.S., Alimov, A.P., Rilo, H.L., Jandacek, R.J., Woollett, L.A., Penberthy, W.T., 2008. A high throughput live transparent animal bioassay to identify non-toxic small molecules or genes that regulate vertebrate fat metabolism for obesity drug development. Nutr. Metab. 5, 23.

Kawai, M., Rosen, C.J., 2010. PPARγa: a circadian transcription factor in adipogenesis and osteogenesis. Nat. Rev. Endocrinol. 6/11, 629–636.

Kawai, M., Green, C.B., Horowitz, M., Ackert-Bicknell, C., Lecka-Czernik, B., Rosen, C.J., 2010. Nocturnin: a circadian target of Pparg-induced adipogenesis. Ann. N. Y. Acad. Sci. 192, 131–138.

Krause, M., Klit, A., Jensen, M.B., Soeborg, T., Frederiksen, H., Schlumpf, M., et al., 2012. Sunscreens: are they beneficial for health? An overview of endocrine disrupting properties of UV-filters. Inter. J. Androl. 35 (/3), 424–436.

Liu, C., Wang, Q., Liang, K., Liu, J., Zhou, B., Zhang, X., et al., 2013. Effects of tris(1,3-dichloro-2-propyl) phosphate and triphenyl phosphate on receptor-associated mRNA expression in zebrafish embryos/larvae. Aquat. Toxicol. 128-129, 147–157.

Pando, M.P., Sassone-Corsi, P., 2002. Unraveling the mechanisms of the vertebrate circadian clock: zebrafish may light the way. Bioessays 24/5, 419–426.

Riu, A., Grimaldi, M., le Maire, M.A., Bey, G., Phillips, K., Boulahtouf, A., et al., 2011. Peroxisome proliferator-activated receptor gamma is a target for halogenated analogs of bisphenol A. Environ. Health Perspect. 119 (/9), 1227–1232.

Schlegel, A., Gut, P., 2015. Metabolic insights from zebrafish genetics, physiology, and chemical biology. Cell. Mol. Life Sci. 72 (12), 2249–2260.

Trasande, L., Zoeller, R.T., Hass, U., Kortenkamp, A., Grandjean, P., Myers, J.P., et al., 2015. Estimating burden and disease costs of exposure to endocrine-disrupting Chemicals in the European Union. J. Clin. Endocrinol. Metab. 100 (4), 1245–1255.

Weger, M., Weger, B.D., Diotel, N., Rastegar, S., Hirota, T., Kay, S.A., et al., 2013. Real-time in vivo monitoring of circadian E-box enhancer activity: a robust and sensitive zebrafish reporter line for developmental, chemical and neural biology of the circadian clock. Dev. Biol. 380/2, 259–273.

WHO (World Health Organization)/UNEP (United Nations Environment Programme), 2013. In: Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T. (Eds.), The State-of-the-Science of Endocrine Disrupting Chemicals - 2012. UNEP/WHO, Geneva.