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Q4 Q1
Exposure to endocrine disrupting chemicals perturbs lipid metabolism and circadian rhythms
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Q3 Q2
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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 24 hr 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. 16–31
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38 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 Pparγ, the central regulator of adipogenesis (Kawai et al., 2010). 38–56

57 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 57–61

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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-3is 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 hour 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

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